Docket No.: 02136/9420908-000
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Ian C. Bathurst et al.

Application No.: 10/535,609 | Confirmation No.: 7257

Filed: November 2, 2007 | Art Unit: 1614

For: COMPOSITION AND METHOD FOR TREATING INFLAMMATORY DISEASES USING PROTEASE INHIBITORS | Examiner: To Be Assigned

THIRD-PARTY SUBMISSION UNDER 37 C.F.R. § 1.99

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 C.F.R. § 1.99, the undersigned hereby submits six references relating to U.S. Patent Application No. 10/535,609 (U.S. Publication No. 2008/0095806) that published on April 24, 2008.

This submission is made within two months from the date of publication, and is accompanied by the $180.00 fee set forth in 37 C.F.R. § 1.17(p).

The six references are identified on Page 2 of this submission, and copies of the references are attached hereto as Tabs 1-6:

TAB

1. U.S. Patent No. 5,217,951 Issued June 8, 1993, TREATMENT OF INFLAMMATION by Inventors John LEZDEY et al.

2. U.S. Patent No. 6,096,327 Issued August 1, 2000, COSMETIC COMPOSITIONS CONTAINING HUMAN TYPE SERINE PROTEASE INHIBITORS FOR REVITALIZING THE SKIN by Inventors John LEZDEY et al.

3. EP 0 512 090 B1 of January 2, 1997, TREATMENT OF INFLAMMATION by Inventors John LEZDEY, et al.

4. Wachter, A.M., and Lezdey, J., "Treatment of atopic dermatitis with alpha$_1$-proteinase inhibitor," *Annals of Allergy*, 69:407-414 (1992).

5. Barszcz, et al, "Alpha$_1$-proteinase inhibitor in psoriasis: reduced activity in symptom-free patients and during flare," *Dermatological Research*, 280:198-206 (1988).

6. Dabbagh, et al, "Alpha-1-Antitrypsin Stimulates Fibroblast Proliferation and Procollagen Production and Activates Classical MAP Kinase Signalling Pathways," *Journal Of Cellular Physiology*, 186:73-81 (2001).

The references listed above are believed to be relevant to examination of the above-identified published patent application, and it is respectfully requested that they therefore be considered by the Examiner during prosecution thereof.

Dated: June 19, 2008

Respectfully submitted,

By_____/Thomas H. Burrows Jr./_____
Thomas H. Burrows Jr.
Registration No.: 60,463
DARBY & DARBY P.C.
P.O. Box 5257
New York, New York 10150-5257
(212) 527-7700
(212) 527-7701 (Fax)

PROOF OF SERVICE

This document, including all attachments was served on the applicant by mailing on June 20, 2008, via First Class Mail, postage pre-paid, to applicant's attorney of record at the following address:

SALIWANCHIK LLOYD &
SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

_____/Thomas H. Burrows Jr./_____
Thomas H. Burrows Jr.
Registration No. 60,463

TAB 1

US005217951A

United States Patent [19]

Lezdey et al.

[11] Patent Number: 5,217,951

[45] Date of Patent: * Jun. 8, 1993

[54] TREATMENT OF INFLAMMATION

[76] Inventors: **John Lezdey**, 976 Kingston Dr., Cherry Hill, N.J. 08034; **Allan J. Wachter**, 9822 S. Grandview, Tempe, Ariz. 85284

[*] Notice: The portion of the term of this patent subsequent to Nov. 24, 2009 has been disclaimed.

[21] Appl. No.: **781,003**

[22] Filed: **Oct. 18, 1991**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 643,727, Jan. 18, 1991, abandoned, which is a continuation-in-part of Ser. No. 598,241, Oct. 16, 1990, abandoned, and a continuation-in-part of Ser. No. 591,630, Oct. 2, 1990, Pat. No. 5,114,917, which is a continuation-in-part of Ser. No. 445,005, Dec. 4, 1989, Pat. No. 5,008,242, which is a continuation-in-part of Ser. No. 242,735, Sep. 9, 1988, abandoned, and a continuation-in-part of Ser. No. 181,707, Apr. 14, 1988, abandoned, Ser. No. 242,735, Apr. 14, 1988, and Ser. No. 181,707, Apr. 14, 1988, each is a continuation-in-part of Ser. No. 946,445, Dec. 24, 1986, abandoned.

[51] **Int. Cl.**[5] **A61K 37/64**

[52] **U.S. Cl.** **514/8**; 514/2; 514/21; 530/395

[58] **Field of Search** 514/2, 8, 21; 530/395

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,916,117	4/1990	Lezdey et al.	514/8
5,008,242	4/1991	Lezdey et al.	514/8

OTHER PUBLICATIONS

Measurement of histamine-releasing factor activity in individual nasal washings: Relationship w/atopy, basophil response, and membrane-bound IgE. Sim et al, J. Allergy Clin. Immunol., Jun. 1992.
Insights In Allergy—vol. 5, No. 1—Apr. 1990.

Primary Examiner—Michael G. Wityshyn
Assistant Examiner—Choon Koh
Attorney, Agent, or Firm—John Lezdey

[57] **ABSTRACT**

A method for the prophylaxis or direct treatment of non-bronchial mast cell implicated diseases or injuries in a patient which comprises administering to the site of the disease or injury an effective amount of at least one serine protease inhibitor, its salts, derivatives or analogs which bind with the mast cells or their mediators.

12 Claims, No Drawings

TREATMENT OF INFLAMMATION

RELATED APPLICATION

This application is a continuation-in-part of application Ser. No. 643,727 filed Jan. 18, 1991, now abandoned, which is a continuation-in-part of application Ser. No. 598,241 filed Oct. 16, 1990, of Lezdey et al, now abandoned, and application Ser. No. 591,630 filed Oct. 2, 1990, now U.S. Pat. No. 5,114,917, which is a continuation-in-part of application Ser. No. 445,005 filed Dec. 4, 1989, now U.S. Pat. No. 5,008,242, which is a continuation-in-part of application Ser. No. 242,735 filed Sep. 9, 1988, now abandoned, and application Ser. No. 181,707 filed Apr. 14, 1988, now abandoned, which are continuations-in-part of application Ser. No. 946,445 filed Dec. 24, 1986, now abandoned.

FIELD OF THE INVENTION

The present invention relates to a method and composition for treating mammals afflicted with non-bronchial mast cell implicated disease. More particularly, the present invention relates to the treatment of certain mast cell implicated non-bronchial diseases, particularly inflammatory conditions in patients, by administering serine protease inhibitors, their analogs, salts or derivatives. There is particularly provided topical compositions for treating the symptoms of inflammatory skin conditions. The inhibitors bind with mast cell and/or T-cell mediators.

BACKGROUND OF THE INVENTION

Prior to the present invention it was generally believed that serine protease inhibitors could be used only to supplement a deficiency occurring as a result of a genetic defect or a chemically produced deficiency resulting from an event such as smoking. Moreover, no consideration was previously given for directly controlling diseases in which mast cells are implicated by administering serine protease inhibitors when serum levels of proteases or protease inhibitors are normal. Mast cells have been found to be implicated in diseases and events such as allergic and non-allergic rhinitis, nasal polyposis, atopic dermatitis, including psoriasis, contact dermatitis, pancreatitis, emphysema, asthma, colitis, Crohn's Disease, wound healing, cluster headaches, coronary artery spasm, etc.

The role of mast cells in humans is the same as in animals. In addition, animals contain counterparts to human α-1-antichymotrypsin, α-1-antitrypsin, and other serine protease inhibitors. In fact, it has been shown that human α-1-antitrypsin will bind with animal mast cells and the mediators derived therefrom.

Inflammation is a non-specific response of tissues to diverse stimuli or insults and results in release of a variety of materials at the site of inflammation that induce pain. It is now recognized that mast cells are implicated in the pathophysiology of inflammatory skin conditions as well as in other physiological disorders. Mast cells provide the greatest source of histamines in acute inflammation. Mast cells have also been noted in hypertropic scars.

Eosinophils, basophils and neutrophils are prominent in inflammatory lesions due to the potent chemoattractants released by mast cells.

Neutrophils are a main source of serine elastase and cathepsin G which are important in the tissue damage of inflammation.

The most direct approach to therapy of inflammatory skin conditions appears to be a direct attack at the site of inflammation of the mediators of inflammation and pain and the reduction of those neutrophilic derivatives which can cause damage to the growth of new tissue during the healing process.

Alpha 1-antichymotrypsin is a plasma protease inhibitor synthesized in the liver. It is a single glycopeptide chain of approximately 68,000 daltons and belongs to a class of serine protease inhibitors with an apparent affinity toward chymotrypsin-like enzymes. Alpha 1-antichymotrypsin is structurally related to alpha 1-antitrypsin.

Alpha 2-macroglobulin is a glycoprotein containing 8–11% carbohydrate which can be isolated from plasma by gel filtration chromatography.

Alpha 1-proteinase inhibitor (alpha 1-antitrypsin) is a glycoprotein having a molecular weight of 53,000 determined by sedimentation equilibrium centrifugation. The glycoprotein consists of a single polypeptide chain to which several oligosaccharide units are covalently bonded. Human alpha-1-proteinase inhibitor has a role in controlling tissue destruction by endogenous serine proteinases. A genetic deficiency of alpha-1-proteinase inhibitor, which accounts for 90% of the trypsin inhibitory capacity in blood plasma, has been shown to be associated with the premature development of pulmonary emphysema. The degradation of elastin associated with emphysema probably results from a local imbalance of elastolytic enzymes and the naturally occurring tissue and plasma proteinase inhibitors. Alpha-1-proteinase inhibitor inhibits human pancreatic and leukocyte elastases. See Pannell et al, Biochemistry. 13, 5339 (1974); Johnson et al, Biochem. Biophys. Res. Commun., 72 33 (1976); Del Mar et al, Biochem. Biophys. Res. Commun., 88, 346 (1979); and Heimburger et al, Proc. Int. Res. Conf. Proteinase Inhibitors. 1st, 1-21 (1970).

The article of Groutas entitled "Inhibitors of Leukocyte Elastase and Leukocyte Cathepsin G Agents for the Treatment of Emphysema and Related Ailments" medical Research Reviews, Vol. 7, No. 7, 227–241 (1987), discloses the role of eglin, elastinal 1 and elasnin in emphysema.

U.S. Pat. No. 4,916,117 to Lezdey et al discloses the treatment of pulmonary inflammation with microcrystalline alpha-1-antichymotrypsin.

It is understood that the term "serine protease inhibitors" as used herein refers to the inhibitors derived from a human source and the corresponding recombinant product which is either glycosylated or non-glycosylated.

SUMMARY OF THE INVENTION

The present invention relates to a method for treating non-bronchial inflammatory conditions in patients by the administration of serine protease inhibitors, their analogs, salts or derivatives which alone or in combination with one or more other serine protease inhibitors which have a specific activity for mast cells or the proteases derived therefrom such as cathepsin-G, elastase, human mast cell chymase, kinins or their precursors in a suitable pharmaceutical composition.

Serine protease inhibitors have been found to play a major role in the direct inactivation of the mediators of

inflammation so that the normal wound healing process can be accelerated without interference from the excess of materials released at the site of inflammation. The almost immediate disappearance of pain and itch indicates that there can be a control of the kinins as well. A cocktail of serine protease inhibitors would therefore be useful to deactivate those mediators of inflammation which may not yet be recognized or are found in association with a particular disease.

As presently found, serine protease inhibitors are useful in the treatment of burn patients which not only experience pain and itch but have a problem in controlling the laydown of organized collagen because of elastase and cathepsin G; serine protease inhibitors permit the rapid growth of normal skin.

The administration of serine protease inhibitors appears to be a viable alternative to the administration of steroids to reduce inflammation and to treat inflammatory skin conditions not treatable with steroids or reduce the steriod requirement.

It has now been found that controlling the amount of the destructive enzymes at the site of inflammation can prevent proliferation of the disease, prevent associated tissue damage and promote healing. It has also been found that the administration of serine protease inhibitors which inactivate destructive proteases alone provide a major control of the symptoms of the disease or burns. However, since the cause of disease may be a result of more than one factors, the use of more than one protease inhibitor provides a better chance of success for early remission of the symptoms and for a prophylactic control of the symptoms associated with the disease. Serine protease inhibitors, for example, alpha 2-macroglobulin, alpha 1-antichymotrypsin and C-reactive protein (CRP), when administered to the site of inflammation provides a reduction in swelling, pain and stiffness.

For chronic cases of dermatitis, a cocktail of protease inhibitors is preferably administered at the site of inflammation. The treatment can be followed with the addition of an appropriate steroid or antibiotic.

The serine protease inhibitors which are contemplated in the present invention are any of the inhibitors, their analogs, derivatives or salts which can inhibit mast cells or bind with any one or more of the protease derived from eosinophils, basophils and/or neutrophils such as elastase, cathepsin-G, tryptase, chymase, kinins, kallikrein, tumor necrosis factor, chymotrypsin, collagenase, and the like.

The serine protease inhibitors included in the present invention are alpha 1-antichymotrypsin, alpha 1-antitrypsin, alpha 2-macroglobulin, alpha 2-antiplasmin, elastinal 1, elasnin 3, C-reactive protein, beta 1-antigellagenase, serine amyloid A protein, alpha cysteine protease inhibitors, inter-alpha-trypsin inhibitor, secretory leucocyte protease inhibitor, bronchial mucous inhibitor, and C-1-inhibitor. The inhibitors of the invention may be natural or prepared by recombinant means.

Alpha 2-antiplasmin is a single-chain glycoprotein containing 11% carbohydrate, and asparagine and leucine as the amino terminal residues. This enzyme has a molecular weight of about 65,000 to 70,000. This inhibitor can inactivate Kallikrein, chymotrypsin (Kass=1.0×10^5 M^{-1} sec^{-1}), plasmin, Factor Xa and Factor XIa.

The use of alpha 1-antitrypsin and alpha 1-antichymotrypsin have been especially useful in the treatment of the various inflammatory skin conditions including those which are induced by autoimmune disease, virus and bacterial infections. The serine protease inhibitors have also been found to cause vasoconstriction, which in inflammation, decreases swelling and redness and to eliminate pain and itching. This feature is especially useful in burns and atopic dermatitis.

Alpha 1-antitrypsin has also been found especially useful in the treatment of topical inflammatory conditions because of its association with elastase. Alpha 1-antitrypsin inhibits glycosylation enhancing factor (GEF) from T-cells so as to prevent degranulation of masts cells by IgE. However, it is preferably used in combination with alpha 1-antichymotrypsin which binds with basophils to inhibit histamine release.

Both alpha 1-antitrypsin and alpha 1-antichymotrypsin alone or in combination control the release of histamines.

The drugs of the invention may be prepared by cloning, by conventional techniques utilizing an oligonucleotide probe or antibody probe, and the like. The recombinant gene product of the invention is especially useful since it is free of contaminating viruses when produced.

The analogs, salts and derivatives may be formed utilizing conventional techniques associated with other proteins without effecting the utility of the compound. There may be prepared the alkali metal salts, acid-addition salts, and esters similar to other proteins or peptides.

Some inflammation conditions are not immediately identifiable as to source and the factors which are involved to produce the different symptoms are not readily apparent. Therefore, it is desirable to administer in some case a combination or cocktail of serine protease inhibitors to provide a broad spectrum of drugs which can provide rapid relief of the different symptoms of inflammation. The most effective combination is alpha 1-antichymotrypsin and alpha 1-antitrypsin and/or alpha 2-macroglobulin. Preferably, the combination is administered in a ratio of 1:1:1: to 3:2:1: either in a single unit or in separate dosage form.

When topically applied, a serine protease inhibitor such as alpha 1-antitrypsin in suitable composition form is useful in the treatment of burns and inflammatory skin diseases such as psoriasis, eczema, acne, and the like. It has been demonstrated that treatment with alpha 1-antichymotrypsin together with α1-antitrypsin has reduced pain when applied to skin lesions.

The use of a non-aqueous lipid miscible carrier, for example, such as prepared with liposomes are particularly advantageous since they provided improved activity at the treatment sites.

The compositions of the invention are preferably administered to patients showing an increase in IgE through a patch or serum test. That is, the patient shows a positive allergic condition.

It is therefore an object of the invention to provide an antiinflammatory composition which can relieve the swelling and redness associated with inflammatory conditions in humans and animals.

It is a further object of the invention to provide an antiinflammatory composition which is well tolerated by the human body and is free of side effects, and for its counterparts for animal use.

It is a yet still further object of the invention to provide a method and a composition for treating inflammatory skin conditions.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The objects of the present invention can be achieved by the administration of serine protease inhibitors alone or in combination in a suitable pharmaceutical form to patients suffering from non-bronchial inflammatory conditions which are associated with mast cell implicated diseases and which includes burns.

The present invention provides a pharmaceutical composition which comprises a compound of this invention and a pharmaceutically acceptable carrier. The compound may be used alone or in combination with other serine protease inhibitors to provide a broad spectrum of treatment.

In the treatment of burns, a 20% solution of a serine protease inhibitor such as α 1-antitrypsin, alone or in combination with other serine protease inhibitors, in sterile water or saline solution, may be sprayed on the patient or the burn area may be wrapped in wet bandages. A wound healing or skin growth factor may be included. The treatment provides immediate relief of pain. The patient may then be treated with the solution daily until the healing process is normal. Depending upon the severity of the burns, the patient may be further treated with other medications to prevent infection.

The following examples further illustrate the practice of this invention, but are not intended to be limiting thereof. It will be appreciated that the selection of actual amounts of specific serine protease inhibitors to be administered to any individual patient (human or animal) will fall within the discretion of the attending physician and will be prescribed in a manner commensurate with the appropriate dosages will depend on the stage of the disease and like factors uniquely within the purview of the attending physician.

EXAMPLE I

A topical cream was prepared as follows:

A. The following mixture was prepared:

α, -antitrypsin	1.0 g
Olive oil	5.0 g
Cetanol	2.0 g
Stearic acid	5.0 g
Glycerin aliphatic acid ester	12.0 g
Tween 60	0.5 g

B. The following mixture was also prepared:

Propylene glycol	0.5 g
Methyl paraben	0.1 g
Propyl Paraben	0.02 g
Purified water	to 100 g in total

The mixture of parts A and B were blended together by conventional means to give a total of 100 g. of 100% by weight topical cream which could be utilized for treatment of acne, eczema, psoriasis, or other inflammatory dermatological conditions. If desired secretory leucocyte protease inhibitor and/or alpha 2-macroglobulin may be added in an amount of 1.0 g to part A.

EXAMPLE II

An olegenous anhyrous ointment was prepared with the following composition:

Composition	%
α, -antitrypsin	1.0
Soy phosphatide	4.0
Plastibase 50W	94.975
Butylated hydroxytoluene	0.025
	100.00

If desired, in lieu of alpha 1-antitrypsin as the active principal, there may utilized the combination of alpha 1-antichymotrypsin and alpha 1-antitrypsin. Other non-aqueous lipid miscible carriers may also be utilized. However, it is understood that other serine protease inhibitors can also be similarly formulated.

EXAMPLE III

1000 mg of PROLASTIN, a composition sold by Cutter Biological, Miles Inc., comprising about 70% α'-antitrypsin and about 10–18% α'-antichymotrypsin was dissolved in 50 ml of saline solution. A patient suffering from atopic dermatitis with swelling and open lesions of the hand was treated by immersing the hand in the solution. Pain disappeared within 6–10 minutes of treatment. Treatment was continued for 1 hour. The redness and swelling disappeared after 1 hour. Twenty four hours after the treatment the lesions were healing without treatment with any other drugs.

A similar composition was utilized as an otic wash for dogs with ear infections followed by the administration of a steriod.

EXAMPLE IV

A suitable cream for topical use was prepared by admixing 43 g of PROLASTIN from Cutter Biological Laboratories, with 6 ml of water and 1000 g of a balm available under the trademark AQUAPHOR, sold by Beiesdorf Inc., Norwalk Conn. AQUAPHOR comprises a mixture of petrolatum, minerial oil, wax and wool wax alcohol.

The cream is useful for minor irritations and in the treatment of inflammatory skin conditions.

EXAMPLE V

In the treatment of colitis a 20% solution with PROLASTIN may be prepared and administered as an enema.

A similar result will be found with an secretory leucocyte protease inhibitor.

We claim:

1. A method for the treatment of non-bronchial mast cell implicated diseases or injury in mammals which comprises administering to the site of the disease or injury an effective amount of alpha 1-antitrypsin, its salt or derivative which has an affinity to mast cells and/or their mediators and T-cell mediators.

2. The method of claim **1** wherein said alpha 1-antitrypsin is recombinant.

3. The method of claim **1** wherein said mast cell implicated disease is dermatitis or psoriasis and said alpha 1-antitrypsin is administered topically.

4. The method of claim **1** wherein said disease is colitis.

5. The method of claim 1 wherein said mammal is an animal.

6. The method of claim 1 wherein said mammal is human.

7. The method of claim 1 wherein said mediators comprise neutrophils and eosinophils.

8. The method of claim 1 wherein said mediators comprise cathepsin G and elastase.

9. The method of claim 1 wherein said mediators comprise kinins.

10. The method of claim 1 wherein said mammal has an elevated IgE level.

11. The method of claim 1 wherein said disease is atopic dermatitis.

12. A method for inhibiting histamine release in a patient suffering from a mast cell implicated disease which comprises administering to said patient an effective amount of alpha 1-antitrypsin, its salts or derivatives which has an affinity to mast cells and/or their mediators and T-cells mediators.

* * * * *

TAB 2

US006096327A

United States Patent [19]

Lezdey et al.

[11] **Patent Number: 6,096,327**

[45] **Date of Patent: Aug. 1, 2000**

[54] **COSMETIC COMPOSITIONS CONTAINING HUMAN TYPE SERINE PROTEASE INHIBITORS FOR REVITALIZING THE SKIN**

[75] Inventors: **John Lezdey**, Voorhees, N.J.; **Allan Wachter**, Tempe, Ariz.

[73] Assignee: **Protease Sciences Inc.**, Clearwater, Fla.

[21] Appl. No.: **09/186,989**

[22] Filed: **Nov. 5, 1998**

[51] **Int. Cl.**[7] **A61K 7/48**

[52] **U.S. Cl.** **424/401**; 424/64; 424/DIG. 5; 514/8; 514/12; 514/21

[58] **Field of Search** ..... 424/401, 64, DIG. 5; 514/844, 845, 846, 8, 12, 21

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,118,707	6/1992	Chatterjee et al.	514/469
5,190,914	3/1993	Lezdey et al.	514/12

Primary Examiner—Jyothsna Venkat

[57] **ABSTRACT**

Cosmetic compositions and methods are provided for revitalizing the skin especially where it is placed in an environment which can cause injury to the skin. The compositions contain an effective amount of a protease inhibitor to provide a prophylactic or repairing effect.

8 Claims, No Drawings

COSMETIC COMPOSITIONS CONTAINING HUMAN TYPE SERINE PROTEASE INHIBITORS FOR REVITALIZING THE SKIN

FIELD OF THE INVENTION

The present invention relates to cosmetic compositions containing human type serine protease inhibitors. More particularly, there is provided cosmetic compositions containing anti-chymase, anti-tryptase and/or anti-elastase protease inhibitors which improves or revitalizes atmosphere damaged skin including chapped lips, wind burn, sun burn and wrinkles resulting therefrom, as well as natural skin eruptions.

BACKGROUND OF THE INVENTION

Alpha 1-antichymotrypsin is a plasma protease inhibitors synthesized in the liver. It is a single glycopeptide chain of approximately 68,000 daltons and belongs to a class of serine protease inhibitors with an apparent affinity toward chymotrypsin-like enzymes. Alpha 1-antichymotrypsin is structurally related to alpha 1-antitrypsin.

Alpha 2-macroglobulin is a glycoprotein containing 8–11% carbohydrate which can be isolated from plasma by gel filtration chromatography.

Alpha 1-proteinase inhibitor (alpha 1-antitrypsin) is a glycoprotein having a molecular weight of 53,000 determined by sedimentation equilibrium centrifugation. The glycoprotein consists of a single polypeptide chain to which several oligosaccharide units are covalently bonded. Human alpha-1 proteinase inhibitor has a role in controlling tissue destruction by endogenous serine proteinases. A genetic deficiency of alpha-1 proteinase inhibitor, which accounts for 90% of the trypsin inhibitory capacity in blood plasma, has been shown to be associated with the premature development of pulmonary emphysema. The degradation of elastin associated with emphysema probably results from a local imbalance of elastolytic enzymes and the naturally occurring tissue and plasma proteinase inhibitors. Alpha-1 proteinase inhibitor inhibits human pancreatic and leukocyte elastases. See Pannell et al, Biochemistry. 13, 5339 (1974); Johnson et al, Biochem, Biophys. Res. Commun., 72 33 (1976); Del Mar et al, Biochem. Biophys. Res. Commun., and Heimburger et al, Proc. Int. Res. Conf. Proteinase Inhibitors. 1st, 1–21 (1970).

SUMMARY OF THE INVENTION

The present invention provides a topical cosmetic composition for improving or revitalizing the texture of skin or as a prophylactic against skin irritations or degradations. The composition is especially useful for treating skin damaged by the atmosphere such as sun damaged or wrinkled skin, chapped lips or skin on face and hands, and for treatment after a chemical peel, for example with a hydroxy glycollic acid or to prevent skin eruptions.

The human type serine protease inhibitors which can be used in the present invention include natural or recombinant alpha 1-antitrypsin, alpha 1-antichymotrypsin, secretory leucocyte protease inhibitor (SLPI), alpha 2-macroglobulin, c-reactive protein, CI-esterase inhibitor and alpha 2-antiplasmin. The most preferred are alpha 1-antitrypsin and alpha 1-antichymotrypsin used alone or in combination.

The wound healing properties of alpha 1-antitrypsin are helpful in cosmetic preparations which are intended to cover blemishes or skin eruption.

The compositions of the invention contain at least about 0.5 percent of the protease inhibitors. The amount of protease inhibitor which generally can be used is about one percent by weight, preferably, about 1 to 10% by weight of composition. Greater amounts can be utilized but are not required to achieve the desired results.

The compositions of the invention can be used in the form of a lotion, creme, gel or solution, depending on the use or treatment contemplated. The extract can be formulated into cosmetic compositions such as lipsticks, hand cremes, after sun compositions, and the like.

The protease inhibitors can be used alone or with other skin treatment compounds such as aloe vera.

It is a general object of the invention to provide a cosmetic composition which contains an effective amount of the protease inhibitor to improve the quality of the skin.

It is another object to provide a cosmetic composition for treating sensitive skins.

It is yet another object to provide a topical composition which helps revitalize environmentally damaged skin.

It is a still further object of the invention to provide a method for improving damaged skin and preventing its occurrence.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides an improvement in cosmetic compositions by providing safe and natural chymase, tryptase and/or elastase inhibitors which are non-irritating to human skin. The anti-viral characteristic of alpha 1-antitrypsin and SLPI are useful in compositions which can transmit viral infections from one user to another exposed to viral infection.

The favorable cosmetic activity of the protease inhibitors is believed to be the results of the chymase, tryptase and elastase inhibition by the protease inhibitors before or during inflammation. Also, the control of the elastase permits the laying down of new tissue without degradation resulting from the presence of the combination of excess elastase and Cathepsin G. After a chemical peel or removal of the upper dermal layer mechanically or naturally, the new tissue layer which is layed down is more resilient and thereby reduces the wrinkles unless scarring or degradation occurs due to excess elastase or cathepsin G. In aging skin, the protease inhibitor appears to revitalize as well as soften the existing skin. The compositions with the protease inhibitor have a prophylactic effect and reduce the incidence of skin eruptions or inflammations as a result of the action against serine proteases or mast cell involvement.

The compositions according to the invention may be presented in all forms normally used for topical application, in particular in the form of aqueous, aqueous-alcoholic or, oily solutions, or dispersions of the lotion or serum type, or anhydrous or lipophilic gels, or emulsions of liquid or semi-solid consistency of the milk type, obtained by dispersing a fatty phase in an aqueous phase (O/VV) or vice versa (VV/O), or of suspensions or emulsions of soft, semi-solid consistency of the cream or gel type, or alternatively of microemulsions, of microcapsules, of microparticles or of vesicular dispersions to the ionic and/or nonionic type. These compositions are prepared according to standard methods.

They may also be used for the scalp in the form of aqueous, aqueous-alcoholic solutions, or in the form of creams, gels, emulsions or foams or alternatively in the form

of aerosol compositions also containing a propellent agent under pressure.

The amounts of the different constituents of the compositions according to the invention are those traditionally used in the cosmetic field.

These compositions constitute, in particular, cleansing, protective, treatment or skin care creams for the face, hands, feet, major anatomical folds or the body (for example day creams, night creams, make-up removal creams, foundation creams, sun-protection creams), fluid foundations, make-up removal milks, protective or skin care body milks, after-sun milks, skin care lotions, gels or foams, such as cleansing or disinfecting lotions, bath compositions, deodorant compositions, aftershave gels or lotions, compositions for treating certain skin disorders such as those mentioned above.

The sun can produce a series of lesions on the skin which can be precancerous (e.g. seborrheic, keratoses or actinic keratoses).

The compositions according to the invention may also consist of solid preparations constituting cleansing bars or soaps.

The compositions may also be packaged in the form of an aerosol composition containing a propellent agent under pressure.

When the composition of the invention is an emulsion, the proportion of the fatty phase can range from 5% to 80% by weight, and preferably from 5% to 50% by weight, relative to the total weight of the composition. The oils, emulsifiers and coemulisifiers used in the composition in emulsion form are chosen from those traditionally used in the cosmetics. The emulsifier and the coemulsifier are present in the composition in a proportion ranging from 0.3% to 30% by weight, and preferably 0.5 to 30% or, better still, from 0.5 to 20%, by weight relative to the total weight of the composition. The emulsion can, in addition, contain lipid vesicles.

When the compositions of the invention is an oily gel or solution, the fatty phase can represent more than 90% of the total weight of the composition.

In a known manner, the composition of the invention may also contain adjuvants which are customary in the cosmetics, such as hydrophilic or lipophilic gelling agents, hydrophilic or lipophilic active agents, preservatives, antioxidants, solvents, perfumes, fillers, screening agents, bactericides, odor absorbers and coloring matter. The amounts of these different adjuvants are those traditionally used in the cosmetic, or dermatological field, and are, for example, from 0.01% to 10% of the total weight of the composition. Those adjuvants, depending on their nature, may be introduced into the fatty phase, into the aqueous phase and/or into lipid spherules.

As oils which can be used in the invention, mineral oils (liquid paraffin), vegetable oils (liquid fraction of shea butter, sunflower oil), animal oils (perhydrosquatene), synthetic oils (Purcellin oil), silicone oils (cyclomethicone) and fluorinated oils (perfluoro polyethers) may be mentioned.

Fatty alcohols, fatty acids (stearic acid) and waxes (paraffin, carnauba, beeswax) may also be used as fatty substances.

As emulsifiers which can be used in the invention, glycerol stearate, polysorbate 60 and the PEG-6/PEG-32/glycol stearate mixture sold under the name Tefose® 63 by the company Gattefosse may be mentioned as examples.

As hydrophilic gelling agents, carboxyvinyl polymers (carbomer), acrylic copolymers such as acrylate/alkylacrylate copolymers, polyacrylamides, polysaccharides such as hydroxypropylcellulose, clays and natural gums may be mentioned, and as lipophilic gelling agents, modified clays such as bentones, metal salts of fatty acids such as aluminum stearates and hydrophobic silica, or alternatively ethylcellulose and polyethylene may be mentioned.

As hydrophilic active agents, proteins or protein hydrolysates, amino acids, polyols, urea, allanloin, sugars and sugar derivatives, water-soluble vitamins, starch and plant extracts, in particular those of Aloe vera may be used.

As lipophilic active, agents, retinol (vitamin A) and its derivatives, tocopherol (vitamin E) and its derivatives, essential fatty acids, ceramides and essential oils may be used. These agents add extra moisturizing or skin softening features when utilized.

The compositions of the invention may include other plant or herbal extracts. For example, there may be utilized extracts of Paraguay tea, Kola and Guarana, which provide a source of methylxanthines, saponius, tannins and glycosides that have been shown to reduce swelling and redness. The extract of Paraguay tea is known as "Mate extract" and is described in the "International Cosmetic Ingredient Dictionary", 5th Edition. Mate extract is commercially available in combination with extracts of Kola and Guarana which is sold by Cosmetic Ingredient Resources of Stamford, Conn. under the trademark "QUENCHT."

Each of mate extract, serine protease inhibitor and aloe vera extract are known to provide anti-inflammatory activity. The anti-elastase and anti-tryptase activity of the protease inhibitor has been shown to provide a synergistic effect in treating skin inflammations including sun burn.

A surfactant can be included in the composition so as to provide deeper penetration of the ingredients. Although natural surfactants are preferred, others such as isopropyl myristate can be used.

U.S. Pat. Nos. 4,916,117; 5,215,965; 5,093,316; 5,217,951, which are herein incorporated by reference, disclose the anti-inflammatory characteristics of serine protease inhibitors.

Alpha 1-antitrypsin and alpha 2-macroglobulin have been demonstrated as having anti-viral activity against a wide variety of viruses including HIV and Herpes Simplex.

Since it is quite common that the same cosmetic compositions are often utilized by more than one person so that disease can be spread, it is advantageous to provide a cosmetic composition which possesses anti-viral characteristics. This need exists in both lipsticks and eyeliners or eye shadows.

The following examples illustrating the compositions of the invention are not intended to limit the scope of the invention. The amounts indicated are by weight percent unless otherwise noted.

EXAMPLE 1

A gel is prepared by admixing the following ingredients.

Ingredient	Wt %
Carbomer 940	4.10
Xantham gum	0.15
Propylene glycol	51.94
Dipropylene glycol	10.00
Ethoxydiglycol	15.00

-continued

Ingredient	Wt %
Dimethylisosorbide	10.00
Aloe Vera gel	8.00
Surfactant	0.05
Alpha 1-antitrypsin	1.76
	100%

This composition is useful to reduce wrinkles or after a chemical skin peel.

In lieu of alpha 1-antitrypsin, alpha 1-antichymotrypsin can be utilized alone or in combination with alpha 1-antitrypsin.

EXAMPLE 2

A gel is prepared by admixing the following ingredients:

Ingredient	Wt %
1. Propylene Glycol	51.94
2. Carbomer 940	2.10
3. Dipropylene glycol	10.00
4. Xanthan gum	0.15
5. Ethoxydiglycol	15.00
6. Dimethylisosorbide	10.00
7. Ascorbic Acid	2.00
8. Chloroxylenol	0.20
9. Linoleamidopropyl PG-diammonium chloride phosphate	1.50
10. Glycereth 4.5 Lactate	2.00
11. Aloe Vera Gel	2.00
12. Alpha 1-antitrypsin	2.00
13. Tetrasodium EDTA	0.10
14. Citric Acid	0.010
15. Cocamidopropyl PG-dimonium chloride phosphate	1.00

Ingredients 1 and 2 are mixed to disperse and form a gel. About 80% of ingredient 3 is mixed with ingredient 4, added to the gel and slightly heated with admixture. The balance of 3 is mixed with ingredients 5–10 and added to the gel. Ingredients 11–15 are then admixed and added to the gel at 38 degrees C. After mixing, the gel is brought to room temperature.

This gel composition can be used as an after-sun treatment.

EXAMPLE 3

A lotion is prepared by admixing the following ingredients:

Ingredient	Wt %
Propylene Glycol Stearate	9.50
Isocetyl alcohol	5.00
PEG-100 Stearate	1.20
Water	69.90
Methyl paraben	0.20
Propylene glycol	13.10
Sorbitan palmitate	0.60
Alpha 1-antitrypsin	6.00
Mate extract	0.50
	100%

The lotion can be used to treat chapped hands.

EXAMPLE 4

An anti-wrinkle cream is prepared by mixing the following ingredients:

Ingredient	Wt %
Glycerol stearate	8.0
PEG-100 stearate	2.0
Cetostearyl alcohol	2.5
Disodium EDTA	0.1
Methyl Paraben	0.1
Propylene glycol	6.0
Sorbitan stearate	0.7
Alpha 1-antitrypsin	2.5
Aloe vera gel	5.0
Water	13.5
	100%

EXAMPLE 5

An after-sun composition is prepared by admixing the following ingredients:

Ingredient	Wt %
Carbomer	2.80
Propylene Glycol	40.05
Disodium EDTA	1.10
Methyl Paraben	0.20
Alpha 1-antitrypsin	2.00
Alpha 1-antichymotrypsin	2.00
Mate extract	0.35
Aloe Vera Gel	52.50
	100%

EXAMPLE 6

A solution according to the invention is prepared by admixing the following ingredients:

Ingredient	Wt %
Ethoxyglycol	15.00
Propylene Glycol	35.00
Water	q.s.
Disodium EDTA	0.10
Alpha 1-antitrypsin	4.50
Aloe Vera Gel	36.75
	100%

EXAMPLE 7

A shampoo is prepared by admixing the following ingredients:

Ingredient	Wt %
C12–15 Pareth-7 Carboxylic Acid	10.0
Isosteareth -6 Carboxylic Acid	5.0
Hexylene Glycol	8.0

-continued

Ingredient	Wt %
Chloroxylenol	0.5
Alpha 1-antitrypsin	2.0
Mate Extract	0.5
Aloe Vera Gel	2.0
Na2 EDTA	0.1
Water	71.9
	100%

The shampoo is useful in the treatment of scalp inflammation or itch.

The shampoo can be used for sensitive scalps which have sensations of purities, that is to say by itching or prickling to different factors such as inflammation triggered by local factors such as soaps, surfactants, erythema, and the like.

EXPERIMENT 1

5 adults over 50 years of age for one week were exposed to the summer sun, swam in a fresh water lake and did not utilize a sunscreen during the day. At the end of each day, each adult applied a commercial suntan lotion (Coppertone®) to one half of the face and to the other half applied the composition of Example 4.

At the end of one week, the faces were examined. On each adult the part of the face which was treated with suntan lotion had a noticeable increase in wrinkles around the eyes and some erythema. The side of the face on which the composition of Example 4 was applied had a reduction in the depth of the wrinkles, the skin was smoother and not erythemous. The greater and more numerous the wrinkles before hand, the greater the visible effect of the treatment.

After three weeks without the use of suntan lotion or the alpha 1-antitrypsin composition, skin peeling occurred over a greater part of the face wherein suntan lotion was applied.

What is claimed is:

1. A method for revitalizing skin and reducing wrinkles in the skin which comprises topically administering a composition containing an effective amount of a human serine protease inhibitor to provide an anti-elastase, anti-chymase or anti-tryptase treatment to the skin and a suitable cosmetic carrier.

2. The method of claim 1 wherein said composition contains at least 0.5 percent by weight of said protease inhibitor.

3. The method of claim 2 wherein said composition contains about 1 to 10% by weight of said protease inhibitor.

4. The method of claim 1 wherein said composition is in the form of a lotion, cream of gel.

5. The method of claim 1 wherein said protease inhibitor is selected from the group consisting of alpha 1-antitrypsin and secretory leucocyte protease inhibitor.

6. The method of claim 5 wherein said protease inhibitor is alpha 1-antitrypsin.

7. The method of claim 1 wherein said carrier comprises aloe vera.

8. A method for revitalizing human skin which has been subjected to a chemical peel which comprises topically administering an effective amount of alpha 1-antitrypsin in a suitable cosmetic carrier to the site of the chemical peel.

* * * * *

TAB 3

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 512 090 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
02.01.1997 Bulletin 1997/01

(21) Application number: **91920172.3**

(22) Date of filing: **26.09.1991**

(51) Int. Cl.[6]: **A61K 38/55**, A61K 31/56

(86) International application number:
PCT/US91/06847

(87) International publication number:
WO 92/06706 (30.04.1992 Gazette 1992/10)

(54) TREATMENT OF INFLAMMATION

BEHANDLUNG VON ENTZÜNDUNGEN

TRAITEMENT D'INFLAMMATIONS

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(30) Priority: **16.10.1990 US 598241**
18.01.1991 US 643727
11.04.1991 US 683620

(43) Date of publication of application:
11.11.1992 Bulletin 1992/46

(73) Proprietors:
- **Lezdey, John**
  Cherry Hill, New Jersey 08034 (US)
- **Wachter, Allan**
  Tempe, Arizona 85284 (US)

(72) Inventors:
- **Lezdey, John**
  Cherry Hill, New Jersey 08034 (US)
- **Wachter, Allan**
  Tempe, Arizona 85284 (US)

(74) Representative: **Onn, Thorsten et al**
AB STOCKHOLMS PATENTBYRA
Box 23101
104 35 Stockholm (SE)

(56) References cited:
EP-A- 0 139 383 **EP-A- 0 432 117**
US-A- 4 916 117

- **BIOLOGICAL CHEMISTRY HOPPE-SEYLER, vol. 371, Suppl., May 1990, Berlin (DE); S. C. DIETZE et al., pp. 75-79**
- **JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 264, no. 35, 15 December 1989, Baltimore, MD, US, pp. 21308-21315; N.M. SCHECHTER et al.: "Reaction of Human Skin Chymotrypsin-like Proteinase Chymase with Plasma Proteinase Inhibitors"**
- **BIOCHEMICAL MEDICINE & METABOLIC BIOLOGY, vol. 38, 1987; FUKUSEN et al., pp. 165-169**
- **JOURNAL OF CLINICAL INVESTIGATION, vol. 71, June 1983; SCHLEIMER et al., pp. 1830-1835**
- **AMERICAN REVIEW OF RESPIRATORY DISEASES, vol. 135, suppl., 1987; WASSERMAN, pp. S46-S48**
- **CLINICAL IMMUNOLOGY & IMMUNOPATHOLOGY, vol. 50, suppl., 1989; MARONE et al., pp. S24-S40**
- **ANNALS OF ALLERGY, vol. 63, no. 6, Suppl., 1989; WASSERMAN, pp. S46-S50**
- **ANNUAL REVIEW OF IMMUNOLOGY, vol. 1, 1983; LARSEN et al., pp. 335-359**

EP 0 512 090 B1

Printed by Rank Xerox (UK) Business Services
2.13.11/3.4

Description

Field of the Invention

The present invention relates to the treatment of mammals afflicted with mast cell implicated disease. More particularly, the present invention relates to the use of serine protease inhibitors, their analogs, salts or derivatives for the preparation of pharmaceutical compositions for the direct or prophylaxis treatment of certain mast cell implicated diseases, particularly inflammatory conditions in patients. There is particularly provided topical compositions for treating the symptoms of inflammatory skin conditions, compositions for treating pulmonary inflammation by inhalation therapy and compositions for treating allergic rhinitis.

Background of the Invention

Prior to the present invention it was generally believed that serine protease inhibitors could be used only to supplement a deficiency occurring as a result of a genetic defect or a chemically produced deficiency resulting from an event such as smoking. Moreover, no consideration was previously given for directly controlling diseases in which mast cells are implicated by administering serine protease inhibitors when serum levels of proteases or protease inhibitors are normal. Mast cells have been found to be implicated in diseases and events such as allergic and non-allergic rhinitis, nasal polyposis, atopic dermatitis, including psoriasis, contact dermatitis, pancreatitis, emphysema, asthma, colitis, Crohn's Disease, wound healing, cluster headaches, coronary artery spasm, rheumatoid arthritis etc.

Inflammation is a non-specific response of tissues to diverse stimuli or insults and results in release of a variety of materials at the site of inflammation that induce pain. It is now recognized that mast cells are implicated in the pathophysiology of inflammatory skin conditions as well as in other physiological disorders. Mast cells provide the greatest source of histamines in acute inflammation. Mast cells have also been noted in hypertropic scars.

It is now recognized that in a certain injury or a disease neutrophils, mast cells, T-cells and their mediators induce an inflammatory state resulting in a localized imbalance of elevated serine proteases with a concominant deficiency of their naturally occurring inhibitors despite normal serine protease inhibitor serum levels. Mast cells are critical in recruiting the cells (eosinophils, basophils and neutrophils) involved in the late phase reaction (LPR). Mast cell and neutrophile mediators appear to have a central role in the LPR. Monocytes through the release of cytokines, interleukin - 1,6 and tumor necrosis factor further amplify the LPR. Platelet activating factor, a mediator from mast cells, neutrophils and platelets is a potent bronchoconstrictor. Histamines are also released by the degranulation of mast cells as well as leukotriene T4 (LTB4) which play an important role in asthma. IgE upon activation by an antagonist causes degranulation of mast cells. Alpha 1-antitrypsin inhibits the mediators of mast cells and neutrophils, and also regulates IgE biosynthesis. The T-cell lymphokine glycosylation enhancing factor (GEF) is a serine protease that has been shown to enhance IgE response. The serine protease inhibitors decrease mast cell mediator release by inhibiting local IgE biosynthesis and T-cell lymphokine production. Serine proteases not only activate kinins and complements but also mediate tissue necrosis. The serine proteases, elastase and cathepsin G, have been shown to stimulate the production of platelet activating factor and LTB4.

Eosinophils and neutrophils are prominent in inflammatory lesions due to the potent chemoattractants released by mast cells.

Neutrophils are a main source of serine elastase and cathepsin G which are important in the tissue damage of inflammation, especially in rheumatoid arthritis.

The most direct approach to therapy of inflammatory skin conditions appears to be a direct attack at the site of inflammation of the mediators of inflammation and pain and the reduction of those neutrophilic derivatives which can cause damage to the growth of new tissue during the healing process.

Alpha 2-macroglobulin is a glycoprotein containing 8-11% carbohydrate which can be isolated from plasma by gel filtration chromatography.

Alpha 1-proteinase inhibitor (alpha 1-antitrypsin) is a glycoprotein having a molecular weight of 53,000 determined by sedimentation equilibrium centrifugation. The glycoprotein consists of a single polypeptide chain to which several oligosaccharide units are covalently bonded. Human alpha 1-proteinase inhibitor has a role in controlling tissue destruction by endogenous serine proteinases. A genetic deficiency of alpha-1-proteinase inhibitor, which accounts for 90% of the trypsin inhibitory capacity in blood plasma, has been shown to be associated with the premature development of pulmonary emphysema. The degradation of elastin associated with emphysema probably results from a local imbalance of elastolytic enzymes and the naturally occurring tissue and plasma proteinase inhibitors. Alpha-1-proteinase inhibitor inhibits human pancreatic and leukocyte elastases. See Pannell et al, Biochemistry. 13, 5339 (1974); Johnson et al, Biochem. Biophys. Res. Commun., 72 33 (1976); Del Mar et al, Biochem. Biophys. Res. Commun., 88, 346 (1979); and Heimburger et al, Proc. Int. Res. Conf. Proteinase Inhibitors. 1st, 1-21 (1970).

The article of Groutas entitled "Inhibitors of Leukocyte Elastase and Leukocyte Cathepsin G Agents for the Treatment of Emphysema and Related Ailments" medical Research Reviews, Vol. 7, No. 7, 227-241 (1987), discloses the

role of eglin, elastinal 1 and elasnin in emphysema.

U.S. Pat. No. 4,732,973 to Barr et al discloses typical analogs of serine protease inhibitors which may be used in the present invention.

U.S. Patent No. 4,916,117 to Lezdey et al discloses the treatment of pulmonary inflammation with microcrystalline alpha-1-antichymotrypsin.

S.C. Dietze et al. in Biol. Chem. Hoppe-Seyler, vol. 371, Suppl. pp 75-79 (May 1990), see especially page 78, suggest that inhibitors of serine proteases with trypsin-like specificity have no effect on degranulation of and histamine release from mast cells. The prejudice expressed by this reference is overcome by the present invention.

EP-A1-0 432 117 by the present inventors refers to the prophylaxis or direct treatment of inflammation by the administering of alpha 1-antichymotrypsin, its salts or derivatives. In the compositions administered, alpha 1-antitrypsin may also be present, but never without the simultaneous presence of the alpha 1-antichymotrypsin.

It is understood that the term "serine protease inhibitors" as used herein refers to the inhibitors derived from a particular species and inhibits the proteases of the same species. However, human serine protease inhibitors may be used in veterinary products but not vice versa.

Summary of the invention

The present invention relates to the use of alpha 1-antitrypsin, secretory leucocyte protease inhibitor, C-reactive protein, serum amyloid A protein and/or alpha-2-macroglobulin, their analogs, salts or derivatives which inhibit the degranulation of mast cells and/or have an affinity to the mediators of mast cells, for the preparation of a pharmaceutical composition for the treatment of diseases implicated by mast cells, neutrophils, T-cells and their mediators.

The diseases especially contemplated are pulmonary diseases, such as cystic fibrosis, asthma or bronchitis, and skin diseases, such as eczema or psoriasis.

In one preferred embodiment, the pharmaceutiocal composition is in the form of an aerosol composition for delivery in the form of microdroplets. This form is primarily intended for the treatment of pulmonary diseases.

In another preferred embodiment, the pharmaceutical composition is in the form of a topical cream, primarily for the treatment of skin diseases.

In a still further preferred embodiment, the pharmaceutical composition includes an effective amount of a corticosteroid.

Serine protease inhibitors have been found to play a major role in the direct inactivation of the mediators of inflammation so that the normal wound healing process can be accelerated without interference from the excess of materials released at the site of inflammation. The almost immediate disappearance of pain and itch indicates that there can be a control of the kinins as well. A cocktail of serine protease inhibitors would therefore be useful to deactivate those mediators of inflammation which may not yet be recognized but are found in association with a particular inflammatory disease.

It is now recognized that in certain injuries or diseases, neutrophils, mast cells, T-cells and their mediators induce an inflammatory state resulting in a localized imbalance of elevated serine protease with a concomitant deficiency of their naturally occurring inhibitors despite normal serine protease inhibitor serum levels. Mast cells are critical in recruiting the cells (eosinophils, basophils and neutrophils) involved in the late phase reaction (LPR). Mast cell and neutrophil mediators appear to have a central role in the LPR. Monocytes through the release of cytokines, interleukin -1,6 and tumor necroses factor further amplify the LPR. Platelet activating factor, a mediator from mast cells, neutrophils and platelets is a potent bronchoconstrictor. Histamines are also released by the degranulation of mast cells as well as leukotriene T4 (LTB4) which play an important role in asthma. IgE upon activation by an antagonist causes degranulation of mast cells. Alpha 1-antitrypsin inhibits the mediators of mast cells and neutrophils, and also regulates IgE biosynthesis. The T-cell lymphokine glycosylation enhancing factor (GEF) is a serine protease that has been shown to enhance IgE response. By also inhibiting GEF there is a two level inhibition in the inflammatory cycle. The serine protease inhibitors decrease mast cell mediator release by inhibiting local IgE biosynthesis and T-cell lymphokine production. Serine proteases not only activate kinins and complements but also mediate tissue necrosis. The serine proteases, elastase and cathepsin G, have been shown to stimulate the production of platelet activating factor and LTB4.

As presently found, serine protease inhibitors are useful in the treatment of burn patients which not only experience pain and itch but have a problem in controlling the laydown of organized collagen because of elastase and cathepsin G; serine protease inhibitors particularly alpha 1-antitrypsin, permit the rapid growth of normal skin without degranulation.

The administration of serine protease inhibitors appears to be a viable alternative to the administration of steroids to reduce inflammation and to treat inflammatory skin conditions not treatable with steroids or to reduce the steriod requirement. However, the combination with a cortiscosteroid has been found to provide a synergistic effect.

It has now been found that controlling the amount of the destructive enzymes at the site of inflammation can prevent proliferation of the disease, prevent associated tissue damage and promote healing. It has also been found that the administration of serine protease inhibitors which inactivate destructive proteases alone provide a major control of

the symptoms of the disease or burns. However, since the cause of disease may be a result of more than one factors, the use of more than one protease inhibitor provides a better chance of success for early remission of the symptoms and for a prophylactic control of the symptoms associated with the disease. Serine protease inhibitors, for example, alpha 2-macroglobulin and C-reactive protein (CRP), when administered to the site of inflammation provides a reduction in swelling, pain and stiffness.

For chronic cases of dermatitis, a cocktail of serine protease inhibitors is preferably administered at the site of inflammation. The treatment can be followed with the addition of an appropriate steroid or antibiotic. There is a synergistic effect when the serine protease inhibitor is used in combination with a corticosteroid.

Among the corticosteroids which may be used in the present invention are triamcinolone acetonide, flurandrenolide, prednisone, amcinonide, dexamethasone, betamethasone valerate, halocinonide, clocortolone, hydrocortisone valerate, and the like.

Serine protease inhibitors have been found to play a major role in the direct inactivation of the mediators of inflammation so that the normal wound healing process can be accelerated without interference from the excess of materials released at the site of inflammation. The almost immediate disappearance of pain and itch indicates that there can be a control of the kinins as well. Serine protease inhibitors, their analogs, salts or derivatives, appears to provide the quickest healing of psoriatic lesions when used in combination with a corticosteroid.

As presently found, serine protease inhibitors are useful in the treatment of chronic psoriasis patients which not only experience pain and itch but have a problem in controlling the laydown of organized collagen because of elastase and cathepsin G; serine protease inhibitors permit healing and the growth of normal skin. The presence of the steroids enhance the healing and promote a more rapid skin growth which is initiated by the serine protease inhibitors.

The serine protease inhibitors which are contemplated in the present invention are any of the inhibitors, their analogs, derivatives or salts of the human type which can inhibit mast cells or bind with any one or more of the protease derived from eosinophils, basophils and/or neutrophils such as elastase, cathepsin-G, tryptase, chymase, kinins, kallikrein, tumor necrosis factor, chymotrypsin, collagenase, inhibit IgE production and the like.

The serine protease inhibitors included in the present invention are human alpha 1-antitrypsin, alpha 2-macroglobulin, eglin, elastinal 1, elasnin 3, eglin 2, C-reactive protein, beta 1-antigellagenase, serum amyloid A protein, alpha cysteine protease inhibitors, inter-alpha-trypsin inhibitor, secretory leucocyte protease inhibitor, bronchial mucous inhibitor, and C-1-inhibitor. The inhibitors of the invention may be natural or prepared by recombinant means. The recombinant may be glycosylated.

The use of alpha 1-antitrypsin has been especially useful in the treatment of the various inflammatory skin conditions including those which are induced by autoimmune disease, virus and bacterial infections. The serine protease inhibitors have also been found to cause vasoconstriction, which in inflammation, decreases swelling and redness and to eliminate pain and itching. This feature is especially useful in burns and atopic dermatitis.

Alpha 1-antitrypsin has also been found especially useful in the treatment of bronchial and topical inflammatory conditions because of its association with elastase.

The drugs of the invention may be derived from human blood or prepared by cloning, by conventional techniques utilizing an oligonucleotide probe or antibody probe, and the like. The recombinant gene product of the invention is especially useful since it is free of contaminating viruses when produced.

The analogs, salts and derivatives may be formed utilizing conventional techniques associated with other proteins without effecting the utility of the compound. There may be prepared the alkali metal salts, acid-addition salts, and esters similar to other proteins or peptides.

Some inflammation conditions are not immediately identifiable as to source and the factors which are involved to produce the different symptoms are not readily apparent. Therefore, it is desirable to administer in some case a combination or cocktail of serine protease inhibitors to provide a broad spectrum of drugs which can provide rapid relief of the different symptoms of inflammation. The most effective combination is alpha 1-antitrypsin and alpha 2-macroglobulin. Preferably, the combination is administered in a ratio of 1:1: to 2:1: either in a single unit or in separate dosage form.

When topically applied, a serine protease inhibitor such as alpha 1-antitrypsin in suitable composition form is useful in the treatment of burns and inflammatory skin diseases such as psoriasis, eczema, acne, and the like.

The use of a non-aqueous lipid miscible carrier, for example, such as prepared with liposomes are particularly advantageous since they provided improved activity at the treatment sites.

The compositions of the invention are preferably administered to patients shoving an increase in IgE through a patch or serum test. That is, the patient shows a positive allergic condition. These allergic patients having asthma respond quickly to therapy with alpha 1-antitrypsin when administered by inhalation form.

The present invention also provides a method for the prophylactic and direct treatment of patients suffering from allergic rhinitis and the symptoms thereof. In accordance with the invention, there is nasally administered to the patient an effective amount of a serine protease inhibitor, its analog, derivative or salt in a suitable pharmaceutically acceptable carrier. The serine protease inhibitors, analog, derivative or salt is one which is capable of binding with a protease in pollen, a protease derived from mast cells, neutrophils or T-cells or decreasing the degranulation of mast cells by inhibiting antagonists such as GEF.

Preferably, the serine protease inhibitor is administered in an aqueous solution comprising 0.1 to 4.5% by weight of the inhibitor. A greater amount can be used but is generally not required.

The serine protease inhibitor binds with a stimulator of IgE synthesis or an inflammatory mediator of mast cell degranulation. These inhibitors further prevent protease from activating complement and kinins which cause the discomfiture associated with the disease.

The term "allergic rhinitis" is understood to include rhinitis medicamentosa, rhinitis sicca and atrophic rhinitis. Preferable are the serine protease inhibitors which have a specific inhibiting activity of mast cells and binding with the proteases derived therefrom such as cathepsin-G, elastase, human mast cell chymase, kinins, and the like. The inhibiting activity may be direct or indirect. It has now been found that controlling the amount of mast cells and their mediators inherently controls the amount of the enzymes at the site of inflammation and prevents proliferation of the condition. Serine protease inhibitors or acute phase reactants, when administered to the site of inflammation provides a reduction in swelling of the sinuses.

In the treatment of burns, a 20% solution of a serine protease inhibitor such as α 1-antitrypsin, alone or in combination with other serine protease inhibitors, in sterile water or saline solution, may be sprayed on the patient or the burn area may be wrapped in wet bandages. A wound healing or skin growth factor may be included. The treatment provides immediate relief of pain. The patient may then be treated with the solution daily until the healing process is normal. Depending upon the severity of the burns, the patient may be further treated with other medications to prevent infection.

The treatment of rheumatoid arthristis can be performed by injection and/or by topical application such as utilizing an occlusive dressing and an aqueous composition of the drug.

The following examples further illustrate the practice of this invention, but are not intended to be limiting thereof. It will be appreciated that the selection of actual amounts of specific serine protease inhibitors to be administered to any individual patient (human or animal) will fall within the discretion of the attending physician and will be prescribed in a manner commensurate with the appropriate dosages will depend on the stage of the disease and like factors uniquely within the purview of the attending physician.

EXAMPLE I

A topical cream was prepared as follows:

A. The following mixture was prepared:

α_1 -antitrypsin	1.0 g
Olive oil	5.0 g
Cetanol	2.0 g
Stearic acid	5.0 g
Glycerin aliphatic acid ester	12.0 g
Tween 60	0.5 g

B. The following mixture was also prepared:

Propylene glycol	0.5 g
Methyl paraben	0.1 g
Propyl paraben	0.02 g
Purified water	to 100 g

in total

The mixture of parts A and B were blended together by conventional means to give a total of 100 g. of 100% by weight topical cream which could be utilized for treatment of acne, eczema, psoriasis, or other inflammatory dermatological conditions. If desired secretory leucocyte protease inhibitor and/or alpha 2-macroglobulin as well as a corticos-

teroid may be added in an amount of 1.0 g to part A.

EXAMPLE II

An olaginous anhydrous ointment was prepared with the following composition:

Composition	%
α_1 -antitrypsin	1.0
Soy phosphatide	4.0
Plastibase 50W	94.975
Butylated hydroxytoluene	0.025
	100.00

Other non-aqueous lipid miscible carriers may also be utilized. The composition may be used in combination with a topical corticosteroid.

Example III

In the treatment of colitis a 20% solution with alpha 1-antitrypsin may be prepared and administered as an enema. A similar result will be found with an secretory leucocyte protease inhibitor.

Example IV

Microcrystalline alpha-1-antitrypsin is suspended in oleic acid and added into a metering aerosol cannister together with trichloromonofluoromethane and dichlorodifluoromethane so that the unit has a molecular proportion of alpha-1-antitrypsin to the propellant between 3:1 and 3:2. The unit delivers a quantity of drug equivalent to 42 mcg. The composition can be used in the treatment of asthma.

Example V

Microcrystalline alpha-1-antitrypsin and alpha-1-antitrypsin is suspended in oleic acid and added into a metering aerosol cannister together with trichloromonofluoramethane and dichlorodifluoromethane so that the unit has a molecular proportion of drug to the propellant between 3:1 and 3:2.

Example VI

A composition for use in treating allergic rhinitis was prepared from the following ingredients.

Ingredient	% wt
α_1-antitrypsin	0.1
10% saline solution	99.8
antioxidant	0.1

Example VII

A pilot study was performed which consisted of a non-blinded trial using α_1-PI at a concentration of 20mg/ml in an aqueous solution in an alternate day schedule in conjunction with a 1% cream of α_1-PI (Stage I) and a 5% cream of α_1-PI for maintenance therapy (Stage II). Prior to enrollment in this trial all 6 patients failed to respond to high potency top-

ical steroids. Safety was gauged by careful clinical monitoring of subjective complaints, objective findings of erythema, edema and serial measurements of blood chemistries and complete blood counts. Wound healing was documented by serial photography. Written informed consent was obtained from each patient.

All six patients showed significant clinical improvement within 6 to 21 days of initiation of alternate-day therapy. α_1-PI stopped pain, pruritis and promoted tissue healing without scarring in all six patients. No adverse side effects of therapy were documented by clinical history, physical exam or by blood studies after 120 days of therapy. The results is seen in Table 1.

Pt. #	Age /sex	Clinical Manifestions	Duration of Illness/ Previous Therapy	Lgth of Aqeous a1-PI Therapy	Therapy Response Time		(Stage II) Maint/ enance Therapy	Relapse Rate
1	54/F	Digits and palms had erythematous, edematous, pruritic ulcerated and fisured Lesions. Open wounds were both weeping Bleeding. Antecubital and popliteal fossae were eczematoid and Lichenified. Decreased range of motion of hands.	4 years Oral Prednisone IM Kenalog High Potency Top. Steroids Antibiotics Antipruritics Moisturizer	45 days	4pain & Pruritis 30 Minutes Irange of motion 24 hours reepith= Day 3 ulcer heal= Day 14		5% cream for 60 days 5% cream & steroid (topical) 47 days No therapy 40 days	0 0 0
2	36/F	Digits and palms were blistering, pruritic, oozing and bleeding. Decreased range of motion in both hands. Left hand had concomitant Lymphangitis with flares of her dermatitis. Mild blistering lesions of feet.	5 years Oral Prednisone IM Kenalog High Potency Top. Steroids Antibiotics Coal Tar Preps Antipruritics Moisturizers	60 days	4pain & pruritis 30 Minutes Irange of motion 24 hours Denude/Exfo l= Day 3 Ulcer heal= Day 30		5% cream 21 days 5% cream & steroid 35 days No therapy 50 days	0 0 0
3	36/M	Dorsum of hand had blistering, weeping, erythematous, edematous and pruritic Lesions. Occasional involvement of chest and arms. Lesions would also go through cycles of crusting.	3 years Oral Prednisone IM Kenalog Antipruritics High Potency Top. Steroids	14 days	4pain & pruritis 30 Minutes Ierythe Day 2 Appear Norm= Day 12		No Therapy 90 days	0

4	34/M	Single Chronic Erythematous, Blistering, scalding and pruritic Lesion on right forearms.	5 years Oral Prednisone Antipruritics High Potency, Topical Steroids	42 days	Ipain & pruritis 3 days Erythema day 4 Normal appearing skin day 6			5% cream 30 days No Topical Steroids No Therapy 20 days	0 • 					
5	32/M	Left hand involvement with fissuring, pruritis, scaling, minimal erythema and edema and decreased range of motion.	10 years Oral Prednisone Moisturizers High Potency Topical Steroids	30 days	Ipain & pruritis 30 Minutes range of motion 24 hours Healed skin 30 days			5% cream No Topical Steroids No Therapy 20 days	0 • 					
6	16/M	Bilateral hand involvement with extensive disease to distal phalanges: fissuring, bleeding, painful and pruritic Lesions. Decreased range of motion of hands.	8 years Oral Prednisone Moisturizers Coal Tar Preps	35 days	Ipain & pruritis 4 days Erythema 7 days fissures healed day 7			5% cream & Topical Steroids 40 days	0 					
		• -Only lab data that falls outside of normal limits is tabulated												

Claims

1. The use of alpha-1-antitrypsin, secretory leucocyte protease inhibitor, C-reactive protein, serum amyloid A protein and/or alpha-2-macroglobulin, their analogs, salts or derivatives which inhibit the degranulation of mast cells and/or have an affinity to the mediators of mast cells, for the preparation of a pharmaceutical composition for the treatment of diseases implicated by mast cells neutrophils, T-cells and their mediators.

2. The use according to claim 1, wherein said disease is a pulmonary disease.

3. The use according to claim 2, wherein said pulmonary disease is characterized by elevated elastase level.

4. The use according to any one of claims 1-3, wherein said pharmaceutical composition is an aerosol composition for delivery in the form of microdroplets.

5. The use according to claim 1, wherein said disease is a skin disease.

6. The use according to claim 5, wherein said skin disease is eczema or psoriasis.

7. The use according to any one of claims 1, 5 or 6, wherein the pharmaceutical composition is a topical cream.

8. The use according to any one of claims 1-7, wherein an effective amount of a corticosteroid is included in the pharmaceutical composition.

Patentansprüche

1. Verwendung von alpha 1-Antitrypsin, sekretorischem Leukozyten-Proteaseinhibitor, C-reaktivem Protein, Serum-Amyloid A-Protein und/oder alpha 2-Makroglobulin, deren Analoga, Salzen oder Derivaten, die die Degranulierung von Mastzellen inhibieren und/oder eine Affinität für die Mediatoren von Mastzellen aufweisen, zur Herstellung einer pharmazeutischen Zusammensetzung für die Behandlung von Erkrankungen, an denen Mastzellen, Neutrophile, T-Zellen und deren Mediatoren beteiligt sind.

2. Verwendung nach Anspruch 1, worin die Erkrankung eine Lungenerkrankung ist.

3. Verwendung nach Anspruch 1, worin die Lungenerkrankung durch einen erhöhten Elastase-Spiegel gekennzeichnet ist.

4. Verwendung nach einem der Ansprüche 1 bis 3, worin die pharmazeutische Zusammensetzung eine Aerosolzusammensetzung zur Verabreichung in Form von Mikrotröpfchen ist.

5. Verwendung nach Anspruch 1, worin die Erkrankung eine Hauterkrankung ist.

6. Verwendung nach Anspruch 5, worin die Hauterkrankung Ekzem oder Psoriasis ist.

7. Verwendung nach einem der Ansprüche 1, 5 oder 6, worin die pharmazeutische Zusammensetzung eine topische Creme ist.

8. Verwendung nach einem der Ansprüche 1 bis 7, wobei eine wirksame Menge eines Corticosteroids in der pharmazeutischen Zusammensetzung enthalten ist.

Revendications

1. Utilisation d' α-1-antitrypsine, d'un inhibiteur de leucocyte-protéase sécrétoire, d'une protéine C-réactive, d'une protéine amyloïde A sérique et/ou d' α-2-macroglobuline, de leurs analogues, sels ou dérivés, qui inhibent la dégranulation des mastocytes et/ou ont une affinité pour les médiateurs des mastocytes, pour la préparation d'une composition pharmaceutique pour le traitement de maladies implicant les mastocytes, les neutrophiles, les cellules T et leurs médiateurs.

2. Utilisation selon la revendication 1, dans laquelle ladite maladie est une maladie pulmonaire.

3. Utilisation selon la revendication 2, dans laquelle ladite maladie pulmonaire est caractérisée par un taux d'élastase élevé.

4. Utilisation selon l'une quelconque des revendications 1-3, dans laquelle ladite composition pharmaceutique est une composition en aérosol pour une administration sous forme de microgouttelettes.

5. Utilisation selon la revendication 1, dans laquelle ladite maladie est une maladie de peau.

6. Utilisation selon la revendication 5, dans laquelle ladite maladie de peau est l'eczéma ou le psoriasis.

7. Utilisation selon l'une quelconque des revendications 1, 5 ou 6, dans laquelle la composition pharmaceutique est une crème à usage local.

8. Utilisation selon l'une quelconque des revendications 1-7, dans laquelle une quantité efficace d'un corticostéroïde est incorporée dans la composition pharmaceutique.

TAB 4

Treatment of atopic dermatitis with alpha$_1$-proteinase inhibitor

Allan M. Wachter, MD and John Lezdey

Alpha$_1$-proteinase inhibitor (α_1-PI), a serine protease inhibitor, was tested for its efficacy for the treatment of recalcitrant atopic dermatitis. Atopic dermatitis affects both children and adults and has no established etiology. We hypothesized that during inflammation there is an excess of serine proteases and a deficiency of their naturally occurring inhibitors at the local site of tissue injury, even though there is a normal serum level of serine protease inhibitors. This pilot study consisted of a nonblinded trial using α_1-PI at a concentration of 20 mg/mL in an aqueous solution in an alternate day schedule in conjunction with a 1% cream of α_1-PI (Stage I) and a 5% cream of α_1-PI for maintenance therapy (Stage II). Before enrollment in this trial all six patients failed to respond to high potency topical steroids. Safety was gauged by careful clinical monitoring of subjective complaints, objective findings of erythema, edema, and serial measurements of blood chemistries and complete blood counts. Wound healing was documented by serial photography. Written informed consent was obtained from each patient. All six patients showed significant clinical improvement within 6 to 21 days of initiation of alternate day therapy. Alpha$_1$-PI stopped pain, pruritus, and promoted tissue healing without scarring in all six patients. No adverse side effects of therapy were documented by clinical history, physical examination, or by blood studies after 120 days of therapy. Atopic dermatitis may be one example where inflammation is due to an imbalance of serine proteases and their naturally occurring inhibitors. We conclude that α_1-PI at a concentration of 20 mg/mL, and as a 1% or 5% cream, is a safe and effective nonsteroidal antiinflammatory agent for the treatment of atopic dermatitis.

INTRODUCTION

Atopic dermatitis is characterized by pruritus, asteatosis, lichenification and an erythematous, papulovesicular rash that may develop into weeping wounds that undergo periods of exacerbation and remission.[1-4] Current therapy is mainly symptomatic, consisting of either oral or topical steroids, antipruritics, coal tar preparations, UV light and food avoidance.[1-4] Steroids produce side effects that include skin atrophy, telangiectasia, purpura, striae and suppression of the hypothalamic-pituitary-adrenal axis when applied to large body surface areas.[5]

Although atopic dermatitis has no established etiology, multiple immunologic abnormalities have been documented:

(a) defective cellular immunity with anergy to recall antigens[6];
(b) quantitative T-cell suppressor deficiency[7,8];
(c) decreased lymphocyte blast cell transformation after phytohemagglutinin (PHA) exposure[9,10];
(d) decreased neutrophilic and monocytic chemotaxis[11];
(e) elevated serum immunoglobulin E (IgE) levels[12-15];
(f) altered beta-adrenergic receptor activity resulting in increased serum histamine levels[16-17]; and
(g) mast cell degranulation.[18-19]

Mast cells play a critical role in inflammation by linking both the humoral and cellular immune systems.[18] Both IgE and non-IgE mechanisms activate mast cells to degranulate their mediators.[19] One class of mediators is chymotryptic proteases bound to heparin in the secretory cytoplasmic granules of mast cells.[20] Other mediators released from mast cells act as chemoattractants for eosinophils and neutrophils.[18,19] This pattern of inflammation mediated by mast cells and their proteases may be one of the common denominators linking atopic dermatitis with asthma, allergic rhinitis, and other mast cell-mediated disorders.

Alpha$_1$-proteinase inhibitor (α_1-PI) is one member of the serine protease inhibitor (serpin) supergene family with broad spectrum activity toward tryptic and chymotryptic proteases.[21] Serpin serum levels are elevated in numerous inflammatory disease states including inflammatory bowel diseases,[22,23] rheumatoid arthritis,[24,25] burn patients,[26,27] reproductive tract diseases,[28] and pulmonary inflammatory diseases.[29,30]

The serpin with the highest naturally occurring plasma concentration (80–213 mg/dL) is α_1-PI. Alpha$_1$-PI is an acute phase reactant protein synthesized by hepatocytes and mononuclear phagocytes with a molecular weight of 52,000 daltons.[31] During states of stress the serum concentration of α_1-PI doubles within two to three days.[32] The spectrum of α_1-PI activity includes its natural target the neutrophil elas-

From the Division of Allergy and Clinical Immunology, Thomas-Davis Medical Center, Tempe, Arizona.

Drug supplied by Cutter Biological Inc.
Received for publication December 26, 1991.
Accepted for publication April 27, 1992.

tase as well as cathepsin G, human mast cell chymase and other serine proteases.[21]

The purpose of this study was to test α_1-PI as an alternative to steroids for the treatment of patients with steroid-resistant atopic dermatitis.

METHODS

Experimental Protocol

Patients with dermatitis refractory to conventional therapy (high potency topical steroids, antipruritics, and moisturizing agents) participated in this nonblinded pilot study. Informed consent was obtained from each patient before entering this study.

Entry Criteria

Atopic dermatitis must have been present for at least 1 year. The diagnosis was made clinically and no skin biopsies were obtained. Only the hands or upper extremities were treated. Each patient had an entry evaluation with a complete clinical history and physical examination. Baseline laboratory tests included complete blood count (CBC), simulated multiple automated chemistry (SMAC-20), erythrocyte sedimentation rate (ESR), antinuclear antibody (ANA), complement levels, and allergy epicutaneous skin tests.

Exclusion Criteria

Patients were excluded if they met the following criteria: age below 12 years, pregnancy, diabetes, cardiac disease, malignancy, chronic infection, hypertension, or any significant abnormality in the baseline studies indicative of a disease unrelated to atopy. Patients could not have received systemic steroid therapy for 3 months or topical steroids for 2 weeks before participation in the pilot study.

Experimental Design

An occlusive dressing of plastic vinyl gloves was used for five of the six (5/6) patients. The remaining patient used an op-site wound kit as an occlusive dressing on his arm. Lyophilized α_1-PI (supplied by Miles, Inc. Cutter Biological) was mixed with injectable sterile water to form an aqueous solution with a final concentration of 20 mg/mL. During Stage I therapy, 6 mL of reconstituted α_1-PI was placed into each glove. For Stage I, α_1-PI was blended into a 1% cream and for Stage II into a 5% cream. The emollient cream base consisted of petroleum, mineral oil, mineral wax, and wool alcohol (Aquaphor by Beiersdorf, Inc.). One gram of lyophilized α_1-PI was mixed with 7 mL of injectable sterile water and then blended into 20 g of Aquaphor for the 5% concentration by anhydrous weight.

Stage I

Treatment with aqueous α_1-PI was performed on alternate days. On the day of aqueous treatment, patients kept the glove or occlusive dressing on for two consecutive hours followed by topical application of 1% α_1-PI cream. This sequence was repeated three times during the day. Nocturnal treatment consisted of continuous 8-hour application of aqueous α_1-PI in an occlusive dressing. Alternate day therapy consisted of 1% α_1-PI cream applied topically 3 times each day. No nocturnal treatment with aqueous α_1-PI was applied on alternate days. Photographs were taken serially to document each patient's progress. Symptoms of burning, flushing, itching, pain and signs of blistering, scaling, edema, and erythema were documented.

Stage II

This part of the study consisted of maintenance therapy with 5% α_1-PI cream with or without a topical steroid (mometasone furoate 0.10% cream, Schering Plough, Inc.). Patients received first only 5% α_1-PI cream for 21–90 days. Next they received mometasone furoate 0.1% cream plus 5% α_1-PI cream for an additional 21–40 days. This was done to test for drug interaction and synergism. Finally, all creams were removed and relapse time and rate were documented.

CASE STUDIES

Six patients, two women and four men, ranging in age from 16 to 54 years with histories of atopic dermatitis were studied. The index case is illustrative as all patients exhibited similar symptoms and clinical findings.

Index Case Study

Patient 2 is a 36-year-old woman with a 5-year history of atopic dermatitis involving hands and feet. Patient 2 complained that her hands were continually painful, pruritic, and would easily ooze and bleed. She also experienced difficulty in extending and flexing digits of both hands. Patient 2 is a smoker with a history of chronic bronchitis and asthma.

During the prior 3 years, patient 2 applied high potency topical steroids to her hands and wore gloves during the day as an occlusive dressing. Treatment by several dermatologists included oral prednisone, high potency topical steroids, coal tar preparations, and moisturizers. Patient 2 claimed she only improved when on high doses (60 mg/day) of prednisone and denied benefit from any topical medications. Patient 2 admitted that when her hands would flare, she frequently developed hand infections with an ascending lymphangitis. These infections were difficult to control and would usually only resolve when her dermatitis would improve. Patient 2 refused further oral steroids due to side effects and was next recommended for Grenz therapy.

Physical examination revealed a 36-year-old woman who presented with bloody bandaged hands. Digits and palms bled bilaterally and were papulovesicular, erythematous, edematous, fissured, scaled, blistered, and ulcerated. Her left hand also had a lymphangitic streak extending to her elbow. Patient 2 had mild involvement of her feet with small blistering pruritic lesions on

her soles. The remainder of her examination was within normal limits.

RESULTS

The clinical summaries of the 6 patients are compiled in Table 1. All patients were resistant to conventional therapies. Each patient was treated topically with aqueous α_1-PI and 1% α_1-PI cream. Only patient 3 did not enter Stage II as his condition resolved after only aqueous α_1-PI therapy.

Objective findings of healing were seen in all patients within six days of therapy including epithelialization, increased range of motion, decreased erythema, and healing of fissures. Complete healing of ulcerated skin, edema, fissuring, and erythema occurred within 30 days in all patients. Subjective complaints of pain and pruritus were relieved in 4/6 patients within 30 minutes. The remaining two patients reported relief of pain and pruritus within 3 days (Table 1).

An instructive example was observed in patient 2 who reported almost immediate relief of pain, pruritus and increased range of motion of her hands. Patient 2 reported an immediate anesthetic and soothing effect upon application of aqueous α_1-PI. This was also experienced by patients 1, 3, and 5. Patient 2 had severe bilateral hand involvement. Initially her right hand was treated and her left hand was untreated as a nonblinded control. The right hand exhibited improvement after 5 days of therapy (Figure 1a), while the left hand (the control hand) continued to fissure and bleed (Figure 1b). Patient 2 refused to withhold therapy from the control left hand and on day 6 began aqueous α_1-PI therapy.

Patient 2's hands developed a scaling exfoliative appearance within three days of drug application. Epithelialization began five days after drug application and nonpruritic skin began to appear beneath the exfoliative tissue. Exfoliation ended after 14 days of drug application at

Table 1. Clinical Findings and Therapeutic Response in Six Patients with Recalcitrant Atopic Dermatitis Treated with Alpha$_1$-Proteinase Inhibitor

Pt. No.	Age, Sex	Clinical Manifestations	Duration of Illness/Previous Therapy	No. Days of Aqueous Alpha$_1$-PI Therapy	Therapy Response Time	Stage II Maintenance Therapy*	Relapse Rate
1	54, F	Digits and palms had erythematous, edematous, pruritic, ulcerated and fissured lesions. Open wounds were both weeping and bleeding. Antecubital and popliteal fossae were eczematoid, lichenified with decreased range of motion of hands.	4 years. prednisone, IM Kenalog, HPTS†, antibiotics, antipruritics, moisturizers.	45	Decreased pain and pruritus 30 minutes. Increased range of motion 24 hours. Epithelialization day 3. Ulcers healed day 14.	5% cream (without steroids) 60 days. 5% cream (with steroids) 47 days. No therapy 40 days.	None None Yes
2	36, F	Digits and hands were blistering, pruritic, oozing and bleeding. Decreased range of motion in both hands. Left hand had concomitant lymphangitis with flares of dermatitis. Mild blistering lesions of feet.	5 years. prednisone, IM Kenalog, HPTS, antibiotics, coal tar preparations, antipruritics, moisturizers.	60	Decreased pain and pruritus 30 minutes. Increased range of motion 24 hours. Exfoliation day 3. Epithelialization day 5. Ulcers healed day 30.	5% cream (without steroids) 21 days. 5% cream (with steroids) 35 days. No therapy 42 days.	None None Yes
3	39, M	Dorsum of hand had blistering, weeping, erythematous, edematous and pruritic lesions. Occasional involvement of chest and arms. Lesions would also go through cycles of crusting.	3 years. prednisone, IM Kenalog, HPTS, antipruritics.	14	Decreased pain and pruritus 30 minutes. Decreased erythema day 2. Skin appears normal day 12.	No therapy 90 days.	None
4	34, M	Single chronic erythematous, blistering, scaling and pruritic lesion on right forearm.	5 years. prednisone, antipruritics, HPTS.	42	Decreased pain and pruritus day 3. Decreased erythema day 4. Skin appears normal day 6.	5% cream (without steroids) 30 days. No therapy 20 days.	None Yes
5	32, M	Left hand involvement with fissuring, pruritus, scaling, minimal erythema and edema and decreased range of motion.	10 years. prednisone, moisturizers, HPTS.	30	Decreased pain and pruritus 30 minutes. Increased range of motion 24 hours. Skin appears normal day 3.	5% cream (without steroids) 35 days. No therapy 20 days.	None Yes
6	16, M	Bilateral hand involvement with extensive disease to distal phalanges, fissuring, bleeding, painful pruritic lesions, and with decreased range of motion of hands.	8 years. prednisone, moisturizers, HPTS, coal tar preparations.	35	Decreased pain and pruritus day 4. Decreased erythema day 7. Fissures healed day 7.	5% cream (without steroids) 30 days. 5% cream (with steroids) 40 days. No therapy 38 days.	None None Yes

* All steroids were applied topically.
† High potency topical steroids.

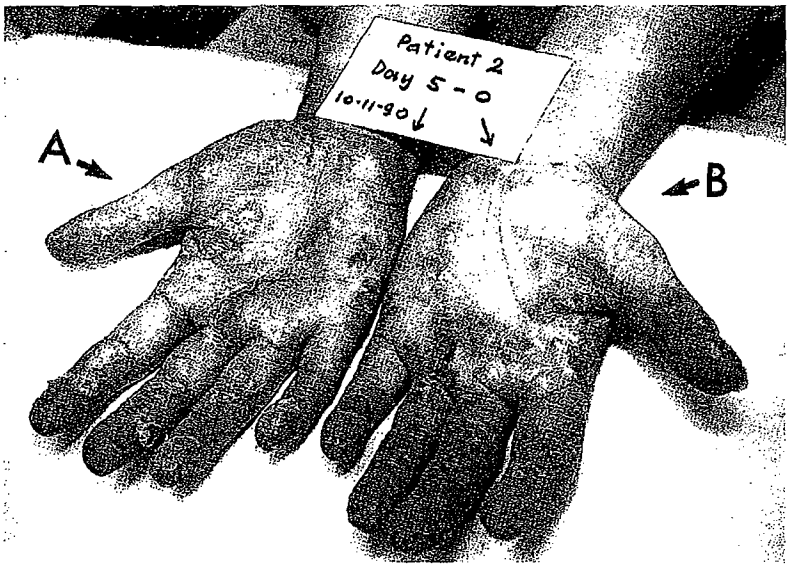

Figure 1. The right hand improved after five days of Stage I therapy. The right hand was less erythematous and edemematous when compared with the left hand (1B). New healthy tissue formed underneath the opened blisters in Figure 1(A). Initially the left hand served as an internal control (untreated) and continued to bleed, fissure, scale, and weep. Because of severe pain and pruritus the patient's left hand also was started on α_1-PI aqueous treatment on day 6.

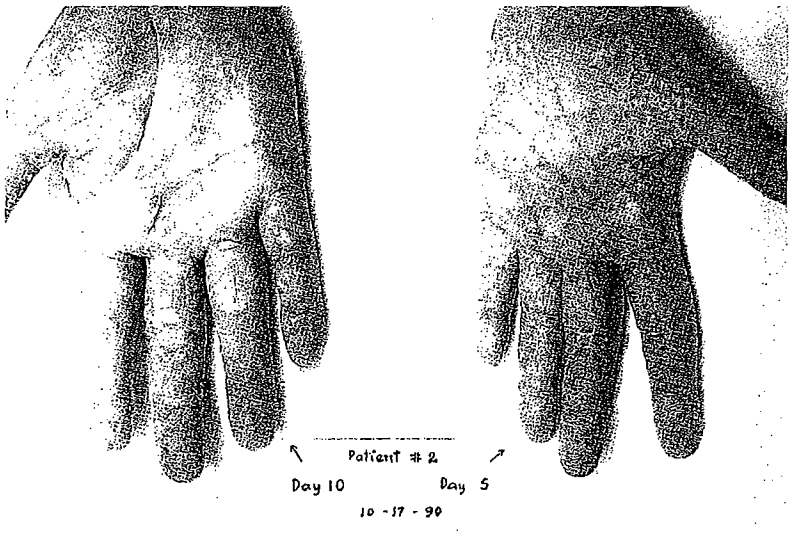

Figure 2. The exfoliative phase began on day 3 for the right hand and day 8 for the left hand. Epithelialization began on day 5 for the right hand. In this picture note palmar exfoliation and decreased edema, erythema, and fissures. There was marked improvement in both hand and digit range of motion by day 10.

which time tissue repair continued (Fig 2). By day 25, there was normal skin with apparent resolution of all clinical manifestations of atopic dermatitis (Fig 3).

Patient 2 was withdrawn from therapy beginning on day 34 to further establish efficacy. Edema, erythema, blisters, and ulcers reappeared within five days of drug withdrawal. Therapy was reinstated on day 39 and patient 2's clinical manifestations began to resolve within five days. Patient 1 also was temporarily withdrawn from Stage I therapy and her dermatitis flared. Stage I therapy was then reinstituted for patient 1 producing results similar to those in patient 2.

Five of six patients entered Stage II for a period of 21 to 90 days without relapse. No patient relapsed with 5% α_1-PI cream alone or in combination with 0.1% mometasone furoate. All five patients relapsed within 20–40 days when removed from Stage II therapy (Table 1). All study patients denied having any local or systemic sensitivity to Stage I or Stage II therapy. No patient evidenced skin irritation, drug induced pruritus, stinging, burning, skin atrophy, striae, hyper- or hypopigmentation, photosensitivity, flushing, or blood chemistry abnormality.

DISCUSSION

We hypothesized that atopic dermatitis is one example where neutrophils, mast cells, T-cells, and their mediators induce an inflammatory state resulting in a localized imbalance of elevated serine proteases with a concomitant deficiency of their naturally occurring inhibitors despite normal serine protease inhibitor serum levels. Severe hand dermatitis is one of the more disabling and difficult skin conditions to treat.[1-3] The exact pathogenesis of atopic dermatitis has been difficult to explain because of the many biochemical and immunologic dysfunctions that have been documented.[6-17] Atopic der-

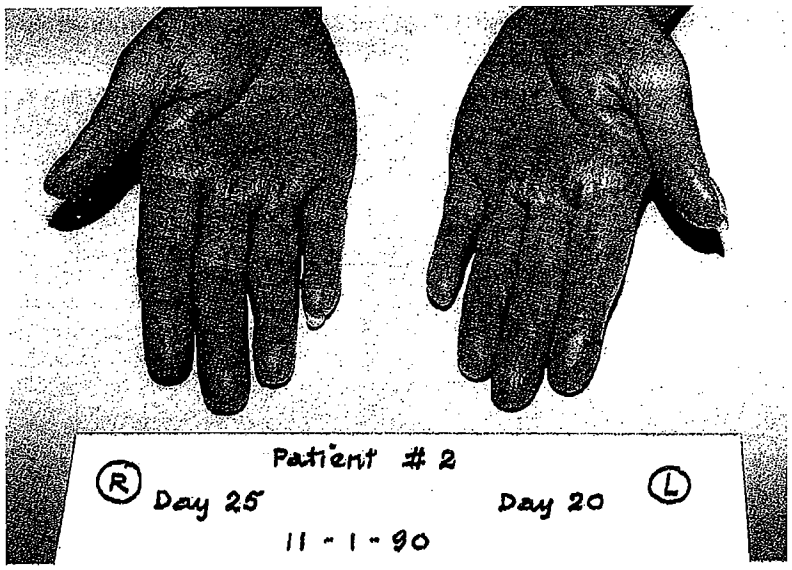

Figure 3. Healing phases, exfoliation and epithelialization were complete for both hands by day 25. All open ulcers and fissures healed without residual scarring or pigmentation. At this stage all clinical symptoms and signs of atopic dermatitis resolved.

matitis is thought to be one example of an uncontrolled allergic late-phase reaction (LPR) seen in skin.[33,34] Although the LPR has been known for many years,[35] only recently have the cells and their mediators been elucidated.[34] Mast cell and neutrophil mediators appear to have a central role in the LPR.[18,19,36] Skin biopsies from patients with atopic dermatitis have increased numbers of mast cells.[37] Compound 48/80 a potent mast cell degranulator induces a LPR in skin.[36,38] Mast cells are critical in recruiting the cells (eosinophils, basophils, and neutrophils) involved in the LPR.[1,2,18,19,33,34,36] Monocytes through the release of cytokines, interleukin-1, 6 (IL-1, 6), and tumor necrosis factor (TNF) further amplify the LPR.[33,39] Platelet activating factor (PAF) a mediator derived from mast cells, neutrophils, and platelets is a potent bronchoconstrictor that produces an intense inflammatory skin response similar to an LPR after intradermal injection.[40,41]

Proteases are released by cells involved in the LPR as well as by natural killer cells and T-cells.[42,43] Alpha$_1$-PI not only inhibits the mediators of mast cells and neutrophils but may also regulate IgE biosynthesis. The T-cell lymphokine glycosylation-enhancing factor (GEF) is a serine protease that has been shown to enhance the IgE response. Alpha$_1$-PI may decrease mast cell and basophil mediator release by inactivating GEF and inhibiting local IgE biosynthesis and histamine release (Fig 4).[44–46] Serine proteases not only activate complement and kinins but also mediate tissue necrosis.[36,47–49] The serine proteases, elastase and cathepsin G, have been shown to stimulate the production of PAF and leukotriene B_4 (LTB_4), mediators of the LPR.[50]

Studies indicate the serine protease inhibitor α_1-PI inhibits PAF and LTB_4 production[33,50,51] (Fig 4). This may be explained by the binding and inactivation of elastase, cathepsin-G and human mast cell chymase by α_1-PI. Alpha$_1$-PI has an association rate constant (K_{ass}) against elastase (K_{ass} 6.5×10^7 m^{-1} sec^{-1}), cathepsin G (K_{ass} 4.1×10^5 m^{-1} sec^{-1}) and human mast cell chymase (K_{ass} 7.5×10^3 m^{-1} sec^{-1}).[21,52] We suggest these serine proteases play an important role in LPR-induced inflammation including atopic dermatitis as well as other neutrophil-, mast cell-, and basophil-mediated disorders. Protease inhibitors may act both at the initial and concluding stages of the inflammatory cascade and their local deficiency may fail to initiate a "shut off signal" necessary to end the cycle of chronic inflammation. Figure 4 demonstrates how α_1-PI and possibly other serine protease inhibitors may modulate the different cell lines and their mediators involved in LPR induced inflammation.[33,34,39–45,50–56]

We tested our hypothesis that atopic dermatitis is a localized imbalance of proteases and antiproteases by applying α_1-PI topically to patients recalcitrant to conventional steroid therapy. All patients enrolled in the pilot study revealed significant improvement in subjective complaints and objective findings after applying α_1-PI topically to the site of inflammation. Topical α_1-PI therapy helped to not only relieve the pruritus and pain of their open sores but healed each patient's wounds without residual scarring.[57–59]

The almost immediate disappearance of pain and pruritus may be explained by the direct inhibition of kallikreins, a class of endogenous serine proteases and their released products, kinins.[36,52,56,60–62] The lack of residual scarring is consistent with the findings that biopsies of scars have increased mast cell numbers and that α_1-PI may bind these mast cell mediators (Fig 4).[37–40,57–59] Scar tissue may be another example of a localized tissue imbalance of proteases and their inhibitors. Efficacy of α_1-PI was further demonstrated when clinical flaring resulted from treatment cessation and resolved with treatment reinstitution.

Four of six patients had open and fissured wounds which may have facilitated drug penetration.

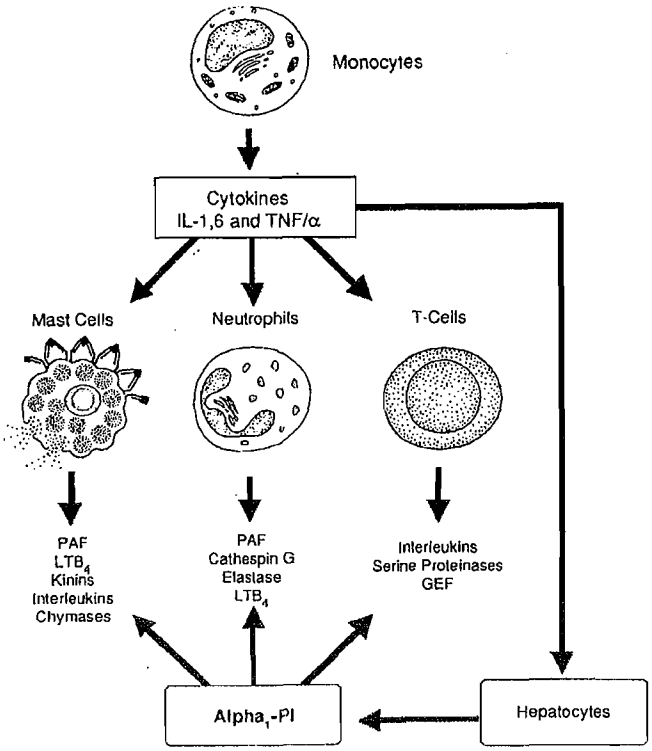

Figure 4. Postulated mechanism of alpha$_1$-PI response to cytokine-induced inflammation. Cytokines activate the cells in the late phase reaction and hepatocytes. Alpha$_1$-PI is primarily a product of hepatocytes which directly inactivates neutrophil elastase, cathepsin G, human mast cell chymase and indirectly limits the production of PAF, LTB_4, T-cell serine proteinases and possibly other mediators of the late phase reaction. This figure demonstrates how cytokines may mediate both the initiation and cessation of inflammation. Abbreviations: LPR: late-phase reaction, IL-1, 6: interleukin 1 and 6, LTB_4: leukotriene B_4, TNF: tumor necrosis factor, PAF: platelet-activating factor, and GEF: glycosylation-enhancing factor.

Drug penetration appeared successful based upon the absence of relapse in 5/5 study patients on Stage II maintenance therapy (Table 1). No studied patient exhibited any clinical, hematologic, or biochemical side effects during Stage I and Stage II therapy. One explanation for the lack of side effects (immune sensitization) is that α_1-PI is a naturally occurring human acute phase reactant that normally rises in serum during disease and states of stress.[21-32] Additionally, α_1-PI binds to specific serine protease mediators of cells involved in the LPR but does not disrupt cellular function in contrast to corticosteroids. Safety of α_1-PI is further substantiated by its 4-year history of approval for parenteral use in congenital α_1-antitrypsin deficiency by the Food and Drug Administration (FDA).

We recognize limitations in this pilot study: small patient sample, absence of a randomized double-blind placebo-controlled trial, absence of pre- and post-treatment biopsies and measurement of local inflamed tissue serine protease and inhibitor levels, and lack of pharmacokinetic studies of α_1-PI topical absorption.

Further trials are warranted to explore the limitations of the present study.

This study suggests the investigation of α_1-PI and other serine or nonserine protease inhibitors, alone or in combination, for their possible synergistic antiinflammatory therapeutic value. Topical administration of α_1-PI may be a viable adjunct or alternative to corticosteroids for the treatment of atopic dermatitis and other inflammatory diseases. This suggests an important role for serine protease inhibitors in blocking the effects of mediators of inflammation.

REFERENCES

1. Hanifin J. Atopic dermatitis. In: Middleton E, Reed CE, Ellis EF, Adkinson NF, Yunginger JW. Allergy principles and practice; 3rd ed. St. Louis: C.V. Mosby Company, 1988:1403–28.
2. Rocklin RE, Pincus S. Atopic dermatitis. In: Samter M, Talmage DW, Frank MM, Austen KF, Claman HN. Immunological diseases, 4th ed. Boston: Little, Brown & Co., 1988:1221–36.
3. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol (Stockholm) 1980; 92 (Suppl):44–47.
4. Sampson HA, McCaskill CC. Food hypersensitivity and atopic dermatitis: evaluation of 113 Patients. J Pediatr 1985;107:669–75.
5. Haynes RC Jr. Adrenocorticotrophic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In: Gilman GA, Rall TW, Nies AS, Taylor P. Goodman and Gilman's the pharmacological basis of therapeutics, 8th ed. New York: Pergamon Press, 1990:1448–52.
6. Palacios J, Fuller EW, Blaylock WK. Immunological capabilities of patients with atopic dermatitis. J Invest Dermatol 1966;47(5): 484–90.
7. Geha RS, Reinherz E, Leung D, et al. Deficiency of suppressor T cells in the hyperimmunoglobulin E syndrome. J Clin Invest 1981;68: 783–91.
8. Beer DJ, Osband ME, McCaffrey RP, et al. Abnormal histamine-in-

duced suppressor-cell function in atopic subjects. N Engl J Med 1982; 306:454–8.
9. Strannegård IL, Lindholm L, Strannegård O. T lymphocytes in atopic children. Int Arch Allergy Appl Immunol 1976;50:684–92.
10. Leung DYM, Wood N, Dubey D, et al. Cellular basis of defective cell-mediated lympholysis in atopic dermatitis. J Immunol 1983;130: 1678–82.
11. Hanifin JM, Rogge JL, Bauman RH. Chemotaxis inhibition by plasma from patients with atopic dermatitis. Acta Derm Venereol (Stockholm) 1980;92(Suppl):52–56.
12. Jones HE, Inouye JC, McGerity JL, et al. Atopic disease and serum immunoglobulin E. Br J Dermatol 1975;92:17–25.
13. Johnson EE, Irons JS, Patterson R, et al. Serum IgE concentration in atopic dermatitis. J Allergy Clin Immunol 1974;54:94–9.
14. Juhlin L, Johansson GO, Bennich H, et al. Immunoglobulin E in dermatoses. Levels in atopic dermatitis and urticaria. Arch Dermatol 1969; 100:12–6.
15. Wittig JH, Belloit J, Fillippi ID, et al. Age-related serum immunoglobulin E levels in healthy subjects and in patients with allergic disease. J Allergy Clin Immunol 1980;66: 305–13.
16. Heskel NS, Chan SC, Thiel ML, et al. Elevated umbilical cord blood leukocyte cyclic adenosine monophosphate-phosphodiesterase activity in children with atopic parents. J Am Acad Dermatol 1984;11:422–6.
17. Szentivanyi A. The beta adrenergic theory of the atopic abnormality in bronchial asthma. J Allergy 1968; 42:203–32.
18. Bernstein JA, Lawrence ID. The mast cell: a comprehensive, updated review. Allergy Proc 1990;11(5): 209–23.
19. Rothe MJ, Vowak M, Kerdel FA. The mast cell in health and disease. J Am Acad Dermatol 1990;23: 615–24.
20. Kido H, Fukusen N, Katunuma N. Chmotrypsin- and trypsin-type serine proteases in rat mast cells: properties and functions. Arch Biochem Biophys 1985;239:436–43.
21. Travis J, Salvesen GS. Human plasma proteinase inhibitors. Annu Rev Biochem 1983;52:655–709.
22. Weeke B, Jarnun S. Serum concentration of 19 serum proteins in Crohn's disease and ulcerative colitis. Gut 1971;12:297–302.
23. Chambers RE, Stross P, Barry RE, et al. Serum amyloid A protein compared with C-reactive protein, alpha$_1$-antichymotrypsin and alpha$_1$-acid glycoprotein as a monitor of inflammatory bowel disease. Eur J Clin Invest 1987;17:460–67.
24. Chard MD, Calvin J, Price CP, et al. Serum α_1-antichymotrypsin concentration as a marker of disease activity in rheumatoid arthritis. Ann Rheum Dis 1988;47:665–71.
25. Panush RS. Human mononuclear cells and neutral proteinase III. Neutral proteinase and rheumatoid arthritis: monocytes as a source of cathepsin G and proteinase potentiation of IgM rheumatoid factor elaboration. Inflammation 1989; 13:359–66.
26. Coombes EJ, Shakespeare PG, Batstone GF. Urine proteins after burn injury. Clin Chim Acta 1979;95: 201–09.
27. Daniels JC, Larson DL, Abston S, et al. Serum protein profiles in thermal burns II. Protease inhibitors, complement factors, and C-reactive protein. J Trauma 1974;14: 153–62.
28. Earl U, Morrison L, Gray C, et al. Proteinase and proteinase inhibitor localization in the human placenta. Int J Gynecol Pathol 1985;8: 114–24.
29. Stockley RA, Burnett D. Alpha$_1$-antichymotrypsin in infected and noninfected sputum. Am Rev Respir Dis 1980;122:81–8.
30. Burnett D, McGillvray DH, Stockley RA. Evidence that aveolar macrophages can synthesize and secrete alpha$_1$-antichymotrypsin. Am Rev Respir Dis 1984;129:473–6.
31. Pannell R, Johnson D, Travis J. Isolation and properties of human plasma α_1-proteinase inhibitor. Biochemistry 1974;13:5439–45.
32. Laurel CB. Pulmonary emphysema and proteolysis. Variation of the alpha$_1$-antitrypsin level of plasma. In: Mittman C, ed. New York: Academic Press, 1972:161–6.
33. Charlesworth EN. Therapeutic implications of late-phase allergic reaction in the skin. Insights In Allergy 1990;5:1–5.
34. Kaliner M. Hypotheses on the contribution of late-phase allergic responses to the understanding and treatment of allergic diseases. J Allergy Clin Immunol 1984;73: 311–5.
35. Cooke RA. Studies in specific hypersensitiveness IX. On the phenomenon of hyposensitization (the clinically lessened sensitiveness of allergy). J Immunol 1922;7: 219–42.
36. Dor PJ, Vervloet D, Sapene M, et al. Induction of late cutaneous reaction by kallikrein injection: comparison with allergic-like late response to compound 48/80. J Allergy Clin Immunol 1983;71: 363–70.
37. Mihm MC, Soter NA, Dvorak HF, et al. The structure of normal skin and the morphology of atopic eczema. J Invest Dermatol 1976; 67:305–12.
38. Atkins P, Green GR, Zweiman B. Histologic studies of human skin test responses to ragweed, compound 48/80, histamine. J Allergy Clin Immunol 1973;51:263–73.
39. Gordon JR, Galli SJ. Mast cells are a source of both preformed and immunologically inducible TNF-α/cachectin. Nature 1990;346:274–6.
40. Basran GS, Page CP, Paul W, et al. Platelet-activating factor: a possible mediator of the dual response to allergen? Clin Allergy 1984;14: 75–9.
41. Archer CB, Page CP, Morley J, et al. Inflammatory characteristics of platelet activating factor (PAF-acether) in human skin. Br J Dermatol 1984;110:45–50.
42. Salvesen G, Farley D, Shuman J, et al. Molecular cloning of human cathepsin G: structural similarity to mast cell and cytotoxic T lymphocyte proteinases. Biochemistry 1987;26:2289–93.
43. Hodig D, Gregg NJ, Kam CM, et al. Lymphocyte granule-mediated cytolysis requires serine protease activity. Biochem Biophys Res Commun 1987;149:882–8.
44. Uede T, Ishizaka K. Formation of IgE-binding factors by rat T-lymphocytes. VI. Cellular mechanisms for the formation of IgE-potentiating factor and IgE-suppressive factor by antigenic stimulation of antigen-primed spleen cells. J Immunol 1982;129:1391–7.
45. Iwata M, Munoz JJ, Ishizaka K.

Modulation of the biologic activities of IgE-binding factor IV. Identification of glycosylation-enhancing factor as a kallikrein-like enzyme. J Immunol 1983;131: 1954–60.
46. Ishizaka K. Perspectives in the application of IgE regulatory factors to the diagnosis and management of allergic diseases. The Proceedings Of The XII International Congress of Allergology And Clinical Immunology. St. Louis: C.V. Mosby Company, 1986:427–30.
47. Roughley PJ, Barrett AJ. The degradation of cartilage proteoglycans by tissue proteinases. Biochem J 1977;167:629–37.
48. Vartio T, Seppa H, Vaheri A. Susceptibility of soluble and matrix fibronectins to degradation by tissue proteinases, mast cell chymase and cathepsin G. J Biol Chem 1981; 256:471–7.
49. Starkey PM, Barrett AJ, Burleigh MC. The degradation of articular collagen by neutrophil proteinases. Biochim Biophys Acta 1977;483: 386–97.
50. Camussi G, Tetta C, Bussolino F, et al. Synthesis and release of platelet-activating factor is inhibited by plasma alpha$_1$-proteinase inhibitor or alpha$_1$-antichymotrypsin and is stimulated by proteinases. J Exp Med 1988;168:1293–1306.
51. Camussi G, Tetta C, Bussolino F, et al. Tumor necrosis factor stimulates human neutrophils to release leukotriene B4 and platelet-activating factor. Eur J Biochem 1989; 182:661–6.
52. Beatty K, Bieth J, Travis J. Kinetics of association of serine proteinases with native and oxidized alpha$_1$-proteinase inhibitor and alpha$_1$-antichymotrpysin. J Biol Chem 1980; 255:3931–4.
53. Schrader JW. Mast cell differentiation and heterogeneity. In: Befus AD, Bienenstock J, Denburg JA, eds. The ontogeny and differentiation of mast cells: a review. New York: Raven Press, 1986:27–43.
54. Costa JJ. Mast cells and cytokines: new insights into the pathogenesis of allergic diseases. Insights In Allergy 1990;5:2–8.
55. Roitt I. The acquired immune response. II. Production of effectors. In: Roitt I, ed. Essential immunology, 7th ed. Oxford: Blackwell Scientific Publications, 1991:129–51.
56. Beatty K, Bieth J, Travis J. Deficiency of alpha$_1$-PI results in congenital emphysema. J Biol Chem 1980;255:2931–4.
57. Kircher CW, Bunce H, Shetlar MR. Mast cell analyses in hypertrophic scars, hypertrophic scars treated with pressure and mature scars. J Invest Dermatol 1978;70:355–7.
58. Cohen IK, Beaven MA, Horákova Z, et al. Histamine and collagen synthesis in keloid and hypertrophic scar. Surg Forum 1972;23: 509–10.
59. Smith CJ, Smith JC, Finn MC. The possible role of mast cells (allergy) in the production of keloid and hypertropic scarring. J Burn Care Rehabil 1987;8:126–31.
60. Christiansen SC, Zuraw BL, Proud D, et al. Inhibition of human bronchial kallikrein in asthma. Am Rev Respir Dis 1989;139:1125–31.
61. Poblete MT, Reynolds NJ, Figueroa CD, et al. Tissue kallikrein and kininogen in human sweat glands and psoriatic skin. Br J Dermatol 1991;124:236–41.
62. Editorial. Kinins and their antagonists. Lancet 1991;338:287–88.

Request for reprints should be addressed to:
Allan M Wachter, MD
Division of Allergy and Clinical Immunology
Thomas-Davis Medical Center
6301 South McClintock Dr
Tempe, AZ 85283

TAB 5

Arch Dermatol Res (1988) 280:198–206

Archives of Dermatological Research

Alpha$_1$-proteinase inhibitor in psoriasis: reduced activity in symptom-free patients and during flare*

D. Barszcz[1], Z. Zarębska[1], M. Glińska-Ferenz[2], S. Jabłońska[2], M. Tigałonowa[2], and W. Gliński[2]

[1] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
[2] Department of Dermatology, Academy of Medicine, Warsaw, Poland

Summary. The aim of this study was to quantitate the active fraction of the α_1-proteinase inhibitor (α_1-PI) in psoriasis. Serum proteinase inhibitory capacity was measured *vs* porcine pancreatic elastase of a known active fraction against its specific substrate (Suc-Ala$_3$-pNA). The inhibitory capacity was determined in 21 symptom-free patients, 134 patients with skin lesions, and 23 healthy volunteers. Alpha$_1$-PI was found to be significantly decreased in symptom-free patients and in those with stationary lesions, in a manner similar to the reduced activity of neutrophil proteinases, elastase, and cathepsin G. The synthesis of α_1-PI was stimulated during the appearance of active psoriatic lesions, but to a much lesser degree in patients with early onset ($\leq$ 21 years) than in patients with late onset of psoriasis ($>$ 21 years). The early onset subgroup differed by a more frequent familial occurrence of psoriasis and a more severe course of the disease. The data indicate that the regulation of the proteinase-α_1-PI system in psoriasis is abnormal and this may contribute to the pathogenesis of the disease. The decreased α_1-PI during flare may be responsible for the disease activity, at least in patients with early onset of psoriasis.

Key words: Psoriasis – Alpha$_1$-proteinase inhibitor – Alpha$_1$-antitrypsin – Serum proteinase inhibitory capacity

An increased proteolytic activity in skin lesions and in psoriatic scales has been reported by several authors [8–10, 22]. The proteases may originate either from the epidermal and dermal cells or from cellular infiltrate, e.g., leukocytes.

Excessive amounts of chemoattractants to leukocytes are generated in psoriatic epidermis [14, 33], and the accumulation of neutrophils in parakeratotic stratum corneum is one of the most characteristic features of psoriasis [5, 29]. Peripheral blood neutrophils have been found to be hyperreactive in several functional tests [31, 37]. At the site of inflammation, activated neutrophils release their proteolytic enzymes which, if not balanced by inhibitors, can activate complement, unmask some hidden antigens, and cause tissue destruction [3, 15, 21, 24].

Furthermore, the activities of elastase and cathepsin G from neutrophils were increased more than twofold in patients with active plaque lesions [12], whereas significant reduction of these enzyme activities was associated with the beneficial effects of peritoneal dialysis and leukapheresis in patients with psoriasis [11, 13]. These data indicate that regulation of proteinases may be of crucial significance in the pathogenesis of the disease.

Biological control of proteases in tissues is mediated by its inhibitors produced locally [e.g., 30] or diffusing from the circulation [35]. One of the most important inhibitors of serine proteinases is α_1-proteinase inhibitor (α_1-PI, α_1-antitrypsin), an acute-phase protein, present at high concentration in a serum and diffusing to tissues [26, 35]. This relatively unspecific inhibitor, binds most rapidly to neutrophil elastase and somewhat more slowly to cathepsin G [1, 35]. Congenitally decreased serum α_1-PI concentration has been linked to genetic variants of the inhibitor, and individuals carrying Z alleles were found to have a pronounced decrease in the plasma α_1-PI, while those with S alleles showed only a slight decrease [6, 26]. A pronounced deficiency predisposes to premature emphysema [35] resulting from damage to the lung elastic fibers caused by leukocyte elastase [7]. An

Offprint requests to: Daniela Barszcz, Ph. D., Department of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, PL-02-532, Warsaw, Poland

* Presented in part at the 4th International Psoriasis Symposium, 6–11 July 1986, Stanford, USA

association with other disease states has also been described [26, 39].

Among patients with psoriasis, an increased frequency of individuals carrying α_1-PI deficiency genes has been reported [2, 16, 23]. However, S allele was not obviously related to the severity of the disease [23] and the frequency of Z and S alleles was too low, compared with the nonpsoriatic population, to explain pathogenesis of the disease [2, 23]. Heng et al. [16] found the Z and S alleles to be associated with severe psoriasis. It has been suggested that the gaps in basal lamina resulting in the basal keratinocyte herniations, revealed by electron microscopy, are induced by proteolytic enzymes [17, 18]. These findings point to the importance of α_1-PI deficiency in the pathomechanism of psoriasis. Other factors, however, than Z and S alleles also seem to contribute to this deficiency.

The purpose of this study was to quantitate active fraction of serum α_1-PI, as measured on pancreatic elastase, in patients with active and stationary psoriasis, in symptom-free patients, and normal controls. Multifactorial analysis was performed to find the correlation, if any, of the changes in α_1-PI activity with the activities of neutrophil elastase and cathepsin G, and with some clinical features, such as disease activity, extent of the lesions, duration of relapse, and age at onset of psoriasis.

Materials and methods

Selection of patients

One hundred fifty-five patients suffering from psoriasis vulgaris (mean age, 39.8 years, range 10–84; 102 males and 53 females) were studied: 134 patients with skin lesions, 21 symptom-free patients (mean age 39.4 years, range 19–64; 13 males and 8 females) as well as 23 healthy volunteers. The latter group was sex- and age-matched with symptom-free patients. Clinical analysis of data was performed according to the criteria of disease activity based on the character of psoriatic lesions, the dynamics of their spreading and, to some extent, on the duration of recurrence [12]. The grade of the disease activity was defined as follows:

A2, active, guttate psoriasis: widely disseminated small papules (not exceeding 5 mm) with pin-point lesions and scarce small plaques; duration of lesions usually up to 1 month after relapse

A1, active plaque psoriasis: predominantly large plaques, peripherally spreading, and some small papules; no pin-point lesions

A0, stationary psoriasis: large plaques persisting longer than 3 months without peripheral spreading, as well as indolent lesions at typical locations (elbows, knees, etc.)

Materials

Elastase from porcine pancreas was the product of Calbiochem-Behring (La Jolla, Calif., USA); trypsin (bovine pancreas, Type III, 2× crystallized) and human plasma α_1-PI (chromatographically prepared) were obtained from Sigma Chemical (St. Louis, Mo., USA). These preparations were used without further purification.

Enzymatic substrates were as follows: N-succinyl-(L-alanine)$_3$-*p*-nitroanilide (Suc-Ala$_3$-pNA; Calbiochem); Nα-benzoyl-DL-arginine-*p*-nitroanilide · HCl (BAPNA) and 4-nitrophenyl 4′-guanidinobenzoate · HCl from Sigma. Solvents used were of spectroscopy grade: acetonitrile (Merck, Darmstadt, FRG), dimethylformamide (BDH, Pool, England), dimethyl sulfoxide (Fluka, Buchs, Switzerland). Triton X-100 was from BDH and Tris (the highest purity grade) was from Sigma.

Measurements of enzyme activity and inhibition

Serum proteinase inhibitory (SPI) capacity was measured on porcine pancreatic elastase using the assay adapted after Travis and Johnson [34]. The procedure was as follows: sera were diluted before testing with 50 m*M* NaCl in 50 m*M* Tris-HCl buffer, pH 8.0; the preincubation mixture (0.3 ml in a carefully washed, dry cuvette) contained 0.15 ml of a stock elastase solution (550 n*M* of active enzyme in 100 m*M* Tris-HCl buffer, pH 8.0), 0.08% Triton X-100, and an appropriate amount of a diluted serum. The mixture was preincubated in 25° C for 3.5 min and then adjusted to 1 ml with 200 m*M* Tris-HCl buffer, pH 8.0 (25° C) and 0.02 ml of the substrate solution (Suc-Ala$_3$-pNA, 7.5 mg/ml in dimethyl sulfoxide). The solution was mixed immediately (zero time) and placed in a thermostated (25° C) spectrophotometer compartment. A residual activity of not inhibited elastase was measured at 410 nm for the first 2 min in 30-s intervals from which the value for 1 min was evaluated. The increase in absorbance for the elastase control without serum was chosen to be about 0.15 per min, and at least three measurements of the control were made for each daily set of serum determinations. The serum proteinase inhibitory (SPI) capacity is expressed as mg elastase inhibited by 1 ml serum. Under the conditions of that test, an increase in the absorbance of 1 unit would be obtained with 550 pmol elastase.

The active fraction of elastase in the preparation used was evaluated according to Beatty et al. [1]. Briefly, trypsin was titrated for active site using the method of Chase and Shaw [4]. This trypsin was then used to titrate the α_1-PI solution which, in turn, served for the titration of elastase. All titrations were performed one after another, to avoid any loss of activity during storage. The following parameters were used for calculations: α_1-PI, A(1%, 280 nm) 5.30, mol. wt. 53,000 [34]; elastase, A (1%, 280 nm) 18.5, mol. wt. 25,000, and for trypsin, A(1%, 280 nm) 14.3, mol. wt. 23,800 [40].

Trypsin activity and inhibition were determined in 0.05 *M* Tris-HCl, 0.05 *M* NaCl, pH 8.0 [1] against BAPNA as the substrate [39]. The reaction mixture (1 ml) contained 0.5 nmol trypsin and 0.78 mg of the substrate. An increase in the absorption was measured at 405 nm. Other details of the procedure were as for elastase measurements.

Results

SPI capacity as related to the disease activity

Mean SPI capacity in the whole group of psoriatics with skin lesions (0.68 mg/ml) was not different from that of healthy subjects (Table 1). However, this was, apparently, a coincidence since the range of SPI capacities in patients was found to be markedly wider, either lower than 2 SD below the mean of controls in 14.2% of cases, or higher than 2 SD above the mean of controls in 16.4% of cases.

When patients with skin lesions were classified according to the criteria of the disease activity, marked differences in SPI capacity between nonactive and active psoriasis were revealed.

In patients with stationary, inactive skin lesions (A0), the mean SPI capacity was significantly lower ($p < 0.01$) than in healthy subjects (Table 1). The A0 group comprised patients with long-lasting inactive lesions at typical locations who rarely suffered from relapses, as well as patients with inactive plaques, who were more prone to flare and some of their lesions had persisted after recent regression of active plaques.

In active guttate psoriasis (A2), the mean SPI capacity was only slightly lower as compared with healthy subjects, whereas in active plaque psoriasis (A1) it was significantly higher ($p < 0.05$). These two groups of patients differed also in the distribution of individual values for SPI capacity. In the A2 group, 37% of the cases, and in the A1 group up to 54% had SPI capacity above the mean of normal controls. These differences highlight the tendency toward increased SPI capacity in active plaque psoriasis. Despite active skin lesions, 7% of the patients with A2, and 12% with A1 had SPI capacity lower than 0.53 mg/ml (2 SD below the mean of controls).

Patients in remission were found to have significantly reduced SPI capacity (0.54 mg/ml, $p < 0.001$) in comparison with both normal controls and patients with skin lesions.

Table 1. Serum proteinase inhibitory capacity in patients with psoriasis and normal controls

Group of patients		Inhibition of elastase by serum (mg/ml)		
		n	Mean ± SD	Range
Patients with skin lesions		134	0.68 ± 0.19[b]	0.27 – 1.43
Disease activity	A2	41	0.66 ± 0.13[b]	0.45 – 1.01
	A1	59	0.74 ± 0.23[a,b]	0.33 – 1.43
	A0	23	0.60 ± 0.14[a]	0.27 – 0.85
Remission		21	0.54 ± 0.08[a]	0.39 – 0.70
Normal controls		23	0.68 ± 0.08	0.54 – 0.83

[a] Significantly different from normal controls (*t*-test: Re, $p < 0.001$; A0, $p < 0.01$; A1, $p < 0.05$)
[b] Significantly different from remission (*t*-test, $p < 0.001$)

The relationship between SPI capacity and neutrophil serine proteinases

Alterations in the mean values of the SPI capacity in patients with psoriasis, correlated positively with the enzymatic activity of neutrophil elastase and cathepsin G (Fig. 1), except that activities of both enzymes were the lowest in stationary psoriasis, whereas SPI capacity was the lowest in remission.

It should be underscored that the activities of neutrophil elastase and cathepsin G were found to be increased in all patients with active skin lesions (A2, A1), whereas the SPI capacity remained still low in some patients (Fig. 2 and range of A2, A1 in Table 1).

SPI capacity as related to the extent of lesions and the duration of relapse

Some positive correlation between the percentage of skin involvement and SPI capacity can be seen in guttate (A2) and stationary (A0) psoriasis (Table 2),

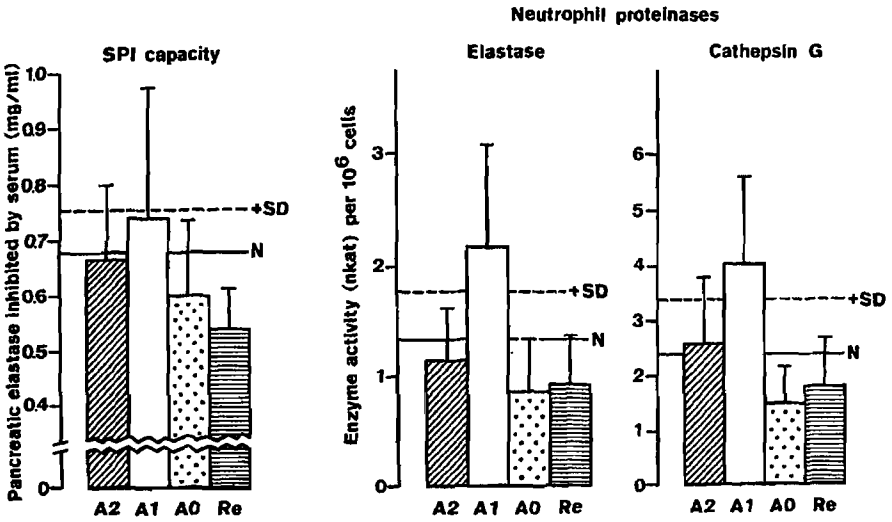

Fig. 1. Comparison between serum proteinase inhibitory (*SPI*) capacity and neutrophil enzyme activities in psoriatics with various disease activity (*A2, A1, A0*) and in symptom-free patients (*Re*). The *horizontal lines* indicate the mean value for normal controls (*N*) and the mean value + SD

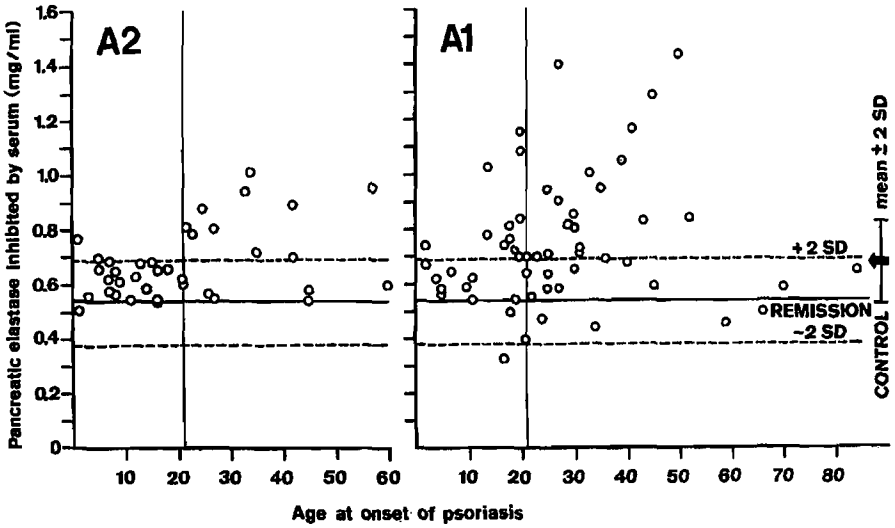

Fig. 2. Serum proteinase inhibitory capacity as related to the age at onset of psoriasis in patients with guttate psoriasis (*A2*) and active plaque psoriasis (*A1*). Each *circle* represents one case. The *horizontal lines* indicate the mean value for symptom-free patients (*REMISSION*) and the mean value ± 2 SD. On the *right margin* are indicated the mean values ± 2 SD for normal controls

Table 2
Serum proteinase inhibitory capacity (mg/ml; mean ± SD) in psoriatics as related to skin involvement

Disease activity	Percent of skin involved				
	< 10	11 – 20	21 – 40	41 – 60	> 60
A2	0.61 ± 0.09 (15)	0.67 ± 0.13 (9)	0.72 ± 0.14 (7)	0.68 ± 0.16 (4)	0.74 ± 0.14 (5)
A1	0.71 ± 0.15 (11)	0.55 ± 0.09 (7)	0.80 ± 0.26 (14)	0.66 ± 0.13 (12)	0.86 ± 0.28 (15)
A0	0.58 ± 0.14 (15)	0.65 ± 0.10 (4)	0.62 ± 0.15 (4)		
Total	0.62 ± 0.13 (43)	0.62 ± 0.12 (20)	0.74 ± 0.22 (28)	0.66 ± 0.14 (16)	0.82 ± 0.26 (21)

The number of cases is given in parentheses

but not in active plaque psoriasis (A1). This suggests that additional factors are affecting SPI capacity.

The duration of recent relapse of psoriatic lesions seemed to have no effect on the mean SPI capacity (data not shown), however some inverse relationship could be noted: the longer the duration of relapse, the lower the inhibitor.

The effect of associated involvement of other organs on SPI capacity in psoriasis

SPI capacity was markedly elevated (0.85 mg/ml) in patients suffering from arthralgia (predominantly those, who had active spreading plaque lesions, A1) as compared with the remaining ones (0.67 mg/ml, Table 3). It was even more elevated in six patients with severe psoriatic arthritis. With the exception of one patient, the highest values were noticed in patients with severe psoriasis and concomitant kidney involvement.

The association between SPI capacity and the age at onset of psoriasis

In patients with active skin lesions, the mean age at the first manifestation of the disease was 24.6 ± 16 years (range, 1 – 84).

An analysis of the SPI capacity in relation to age at onset suggested the existence of two subpopulations: the first with an early onset (⩽ 21 years) and the second with a late onset (> 21 years). This was evident in patients with guttate psoriasis (Fig. 2, A2). There were no patients with the onset at below 21 years of age and SPI capacity close to 0.83 mg/ml (mean of controls + 2 SD). In this subgroup, the mean SPI capacity (0.62 ± 0.07 mg/ml) was significantly lower ($p < 0.001$) than in patients with the same disease activity and a late onset (0.76 ± 0.16 mg/ml). A similar tendency to low SPI capacity in patients with an early onset was seen in the A1 group, although several patients with onset between 14 and 20 years had very high SPI capacity. These patients had, however, widespread lesions (80% – 95%) associated with severe ar-

Table 3. Serum proteinase inhibitory capacity (mean ± SD) as related to joint involvement and kidney disorders in psoriatic patients

Disease activity	Inhibition of elastase by serum (mg/ml)			
	Total	A2	A1	A0
Patients without joint symptoms	0.66 ± 0.14 (78)	0.67 ± 0.12 (26)	0.67 ± 0.16 (37)	0.63 ± 0.13 (10)
Arthralgia and mild arthritis	0.71 ± 0.23 (35)	0.66 ± 0.14 (10)	0.85 ± 0.26 (14)	0.61 ± 0.15 (6)
Severe arthritis	0.97 ± 0.26 (6)[a]			
Kidney disorders	1.05 ± 0.29 (9)[b]			

The number of cases is given in parentheses

[a] The activity of psoriasis in these patients was as follows: A1, in four patients, A2 and Re, each in one patient. Three of the patients with A1 suffered also from kidney disorders

[b] The following kidney disorders were diagnosed in these patients: proteinuria, nephrosis, amyloidosis, glomerular nephritis. The activity of psoriasis was as follows: A1, in eight patients and A0 in one patient

Table 4. Some features of psoriasis in patients with an early (≤ 21 years) and a late (> 21 years) onset of the disease

		Onset of psoriasis at age	
		≤ 21 years	> 21 years
Age at onset (years)		13.0 ± 6.4 (61)	36.6 ± 12.7 (64)
Age at time of study (years)		29.4 ± 12.0 (51)	48.9 ± 15.4 (49)
Duration of relapse (months)		3.6 ± 6.5	4.1 ± 6.3
Extension of lesions (% of skin involved)		32.6 ± 29.2	31.4 ± 26.1
Activity of disease	A2	44.2%[a]	25.0%
	A1	48.1%	53.3%
	A0	7.7%	21.7%[a]
Familial occurrence of psoriasis	Positive	53.3%[a]	25.4%
	One relative	23.3%	17.5%
	More than one	30.0%[a]	7.4%
	First relative[b]	46.7%[a]	20.6%
Psoriatic arthritis	Mild	24.6%	33.8%
	Severe	3.5%	6.5%

The number of cases is given in parentheses. [a] Frequency (χ^2 test) significantly higher ($p < 0.01$). [b] As in Table 5

thritis and/or kidney disorders or arthralgia. Despite this, in patients with A1 and early onset, the mean SPI capacity was lower (0.69 mg/ml) than that of patients with late onset (0.79 mg/ml).

These differences in SPI capacity were not affected by the age of patients at the time of the study. When patients of both subgroups were age-matched (age range, 25–51 years), SPI capacity in patients with the early onset (0.63 ± 0.16) and the late onset (0.75 ± 0.18) were almost the same as given previously for the whole series (cf. Table 5).

Some other clinical data regarding these two subpopulations are presented in Tables 4 and 5. No significant difference has been found in the mean incidence of joint involvement, duration of relapse, and extension of lesions (Table 4). There was, however, better correlation between SPI capacity and the percentage of skin involvement in patients with late onset ($r = 0.42$) as compared with patients with early onset ($r = 0.14$).

There were more cases of guttate psoriasis (44.2%) and markedly less of stationary psoriasis (7.7%) in the "early onset" subpopulation (Table 4). All four patients with stationary lesions in this subpopulation had had previous relapses and in two of them there was a familial history of psoriasis.

Table 5. Serum proteinase inhibitory capacity (mean ± SD) as related to the age at onset of psoriasis

Factor tested		Age at onset of psoriasis	
		⩽ 21 years	> 21 years
Patients with active lesions		0.65 ± 0.15[a] (51)	0.78 ± 0.22 (49)
Disease activity	A2	0.62 ± 0.07 (23)	0.76 ± 0.16 (15)
	A1	0.69 ± 0.19 (25)	0.79 ± 0.26 (32)
	A0	0.64 ± 0.11 (4)	0.61 ± 0.13 (13)
Familial occurrence of psoriasis	None	0.62 ± 0.10 (25)	0.78 ± 0.25 (35)
	One relative	0.69 ± 0.19 (10)	0.83 ± 0.11 (9)
	More than one	0.67 ± 0.17 (16)	0.64 ± 0.04 (5)
	First relative[b]	0.69 ± 0.17 (23)	0.75 ± 0.12 (11)
Female		0.62 ± 0.08 (18)	0.66 ± 0.15 (12)
Male		0.67 ± 0.17 (33)	0.81 ± 0.23 (37)
Duration of relapse	Up to 3 months	0.62 ± 0.10 (26)	0.79 ± 0.21 (23)
	Longer than 3 months	0.68 ± 0.17 (18)	0.72 ± 0.23 (20)

The number of cases is given in parentheses
[a] Pancreatic elastase (mg) inhibited by 1 ml serum
[b] In all but three cases, one close relative was affected (parent, offspring, sibling)

The patients with early onset had more frequent familial occurrence of psoriasis (53.5%) than those with late onset (25.4%, Table 4). Moreover those, who had more than one relative affected, had also decreased SPI capacity even by late onset (Table 5). In the females, the mean SPI capacities were lower and the difference between the mean values was smaller than in the male patients (Table 5).

Discussion

The synthesis of α_1-PI is stimulated by bacterial infection, surgery, etc., as an acute phase protein [26, 39], however, changes in α_1-PI activity in a course of chronic inflammation, such as psoriasis, are less explored.

A quantitation of serum α_1-PI, measured as serum inhibitory capacity against porcine pancreatic elastase hydrolysing its specific low molecular substrate, Suc-Ala_3-pNA, appeared to be a convenient method to determine active α_1-PI. The use of elastase instead of trypsin reduces binding of the enzyme with other serum factors. This enzyme, in contrast to trypsin, is not inactivated by oxidized α_1-PI [1] and other serum inhibitors, such as α_2-antiplasmin, antithrombin III, inter-α-trypsin [35]. In our studies, 1 ml of normal human serum had the capacity to inhibit 0.68 mg (27.2 nmol) of active pancreatic elastase. Based on a 1:1 molecular ratio of enzyme-inhibitor complex, the serum concentration of active α_1-PI was evaluated as 1.44 mg/ml. This value is in the range of 1.34 mg/ml [20] and 1.89 mg/ml [25], both found by immunological methods.

In our studies, SPI capacity was found to be significantly reduced in a vast majority of both symptom-free psoriatics and patients with stationary lesions. A low SPI capacity in remission was also found in patients, who had had the inhibitor increased previously, at the active stage of the disease (data not shown). The decreased α_1-PI activity in serum either could have genetic background or it might be a transient regulatory phenomenon occurring after the regression of psoriatic lesions. The latter is less likely, since remission in our patients lasted from at least 2 weeks up to 3 months.

Alternatively, if decreased α_1-PI activity were genetically determined, it could not be explained solely by the presence of Z and S alleles. We have found α_1-PI activity to be lower than expected for MS phenotypes (smallest genetic deficiency) in 62% of symptom-free patients, in 39% of the patients with stationary psoriasis, and also during flare (12% in A2, 14% in A1), whereas incidence of Z and S alleles in the psoriatic population has been reported as 11.1% [23], 15% [2], and 17.6% [16].

It is possible that decreased serum α_1-PI activity is a characteristic feature of symptom-free psoriasis, as is decreased neutral proteinase of neutrophils [12]. Thus, the equilibrium of the proteinase-inhibitor system would be established in psoriasis at a level lower than in the normal population.

Our data suggest that for any clinical analysis in psoriasis, the reference value should be a mean α_1-PI activity in remission, and not that of the normal population.

When compared to symptom-free psoriasis, patients with active lesions (A2, A1) were found to

have markedly enhance mean α_1-PI activity. Other authors found serum antitrypsin capacity increased over normal value in some patients with active psoriasis [16, 23, 27]. We found, in addition, that this response to inflammation was much lower in patients with an early onset of psoriasis ($\leqslant 21$ years) and in cases with more than one relative affected with the disease. These two subgroups partly overlap, since 76% of the latter cases had the early onset. Furthermore, in females with psoriasis the response to flare seemed to be smaller than in males (0.63 ± 0.13 and 0.71 ± 0.21, respectively; cf. Table 5).

In the subpopulation with the early onset, the majority of the cases (88% in A2, and 54% in A1) showed SPI capacity within the range of remission, despite their extensive and spreading lesions. In addition, a high frequency of flares (more than once a year) was noticed in 50% of these patients, and their lesions were more resistant to therapy. It is conceivable that low α_1-PI during flare contributes to more extensive and active lesions [16–18].

During flare the patients seemed to have a disturbed balance between proteolytic enzymes and their inhibitors. Their SPI capacity was low, whereas neutrophil proteinases (elastase, cathepsin G) were elevated more than twofold in active psoriasis and more granule enzymes were presumably released from migrating neutrophils in tissue. The imbalance may result from the overconsumption of α_1-PI by proteinases with simultaneous insufficient synthesis of α_1-PI. However, it has not yet been established whether the early onset of psoriasis may be due to insufficient protection against the action of neutral proteinases.

It is likely that compensatory regulation of α_1-PI in the late onset subgroup is also defective, however to a lesser extent than in the early onset group. Although the former group included some patients with decreased α_1-PI activity, it consisted mostly of patients able to enhance markedly α_1-PI upon inflammatory stimulation during flare, in contrast with the early onset subpopulation.

The absolute increase in α_1-PI activity by 50% – 100% over mean value in remission argues for the defective regulatory response such as observed in patients with genetic moderate deficiency of α_1-PI [26]. Individuals without genetic defect, after some acute inflammation had up to threefold increased α_1-PI [26].

The patients with late onset had less severe psoriasis and less frequent relapses. There were fewer cases with eruptive guttate psoriasis and more cases with stationary lesions. Thus, psoriasis in these patients may be a self-limiting disease accompanied by a decrease in elastase, cathepsin G, and α_1-PI activities as in stationary psoriasis. However, in this group, there were only slightly fewer patients with more than 40% of skin involvement and more patients (mainly from the A1 group) with inflammation signs from other organs.

The existence of two distinct subpopulations of patients with psoriasis, differing by age of onset, was reported by Henseler and Christophers [19] who also linked a prevalence of the HLA-Cw6 to early onset of psoriasis. These authors suggested that the patients with early onset had an inheritable type of the disease, genetically different from the non-inheritable, with onset in the 5th or 6th decade of life. Our findings on abnormal SPI capacity as well as a higher frequency of familial occurrence of psoriasis in the early onset subpopulation provide further support for their conclusion.

Our results differ from the data of Heng et al. [16], who found a higher serum antitrypsin capacity in mild psoriasis than in the severe form. According to these authors, this was in agreement with a high frequency of α_1-PI deficiency genes in the latter group. The discrepancy between these data and our findings may result either from the selection of patients or from the methodology. These authors selected patients by only one criterion: the percentage of skin involved with psoriatic lesions, where "mild" psoriasis had up to 10% of skin affected, and "severe" more than 20% and this, according to our data, did not correlate directly with α_1-PI activity. Their "mild" and "severe" psoriasis could include patients with both active and inactive skin lesions, which makes any clinical comparison difficult. Moreover, we have found that SPI capacity, measured on trypsin, differs from that determined on elastase, in a way related to the disease activity, at least in some patients with psoriasis (unpublished).

Activated neutrophils [31, 32, 37] and their neutral proteinases [11–13] play an important role in the pathogenesis of psoriasis. They cause damage to the basement membrane of the epidermis [24] as well as change the antigenicity of the stratum corneum [3], thus, neutrophil enzymes might be responsible for perpetuation of the disease.

All these phenomena occurring in the psoriatic skin might be enhanced by the decreased α_1-PI activity, which can result from: (a) decreased α_1-PI concentration in the peripheral blood and/or decreased synthesis of α_1-PI isoforms characteristic for inflammation [36]; (b) inactivation of α_1-PI by oxidation either in the vicinity of stimulated neutrophils [32] or by long-lived reactive intermediates in the distance from neutrophils [38]; (c) local depletion of α_1-PI after binding with elastase and cathepsin G released in excess from neutrophils; (d) proteolytic inactivation of α_1-PI by other proteinases, not inhibited by α_1-PI, e.g., cysteine proteinases [34]; and (e) poor supply of

the inhibitor from other sources, such as macrophages and monocytes, which also synthesize and secrete α_1-PI upon activation [28]. The proper ratio of these cells to neutrophils at the site of inflammation might be of importance in the regulation of the inflammatory process.

In consequence, neutrophil serine proteinases can escape from control and attack susceptible substrates in close proximity to degranulating neutrophils, thus maintaining the inflammation.

In conclusion, it is possible that due to a defective proteinase-proteinase inhibitor system in psoriasis, both α_1-PI and neutral proteinase activities are decreased in remission and in stationary disease. This might be a characteristic genetic feature of psoriasis. During flare, the control of excessively active serine proteinases by α_1-PI might be insufficient and imbalanced as suggested by low stimulation of α_1-PI production in patients with early onset and high familial incidence of the disease.

Acknowledgements. The authors are much indebted to Dr. M. Kocięcki and Dr. W. Król for writing out a computer program for the statistical analysis and gratefully acknowledge the excellent technical assistance of Janina Kalinowska, B. Sc. These investigations were supported by the Polish Academy of Sciences within projects CPBR 3.13.3.2.4 (C-105) and CPBP 06.01.03.

References

1. Beatty K, Bieth J, Travis J (1980) Kinetics of association of serine proteinases with native and oxidised α_1-proteinase inhibitor and α_1-antichymotrypsin. J Biol Chem 255:3931–3934
2. Beckman G, Beckman L, Lidén S (1980) Association between psoriasis and the α_1-antitrypsin deficiency gene Z. Acta Derm Venereol (Stockh) 60:163–164
3. Beutner EH, Binder WL, Jabłońska S, Kumar V (1981) Nature of stratum corneum autoantibodies, antigens and antigen conversion and their role in healing and in psoriasis. In: Marks R, Christophers E (eds) The epidermis in disease. MTP Press, Lancaster, England, pp 333–357
4. Chase T, Shaw E (1970) Titration of trypsin, plasmin, and thrombin with *p*-nitrophenyl *p'*-guanidinobenzoate HCl. In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 19. Academic Press, New York London, pp 20–27
5. Chowaniec O, Jabłońska S (1979) Pre-pin-point papules changes predicting pin-point lesions of psoriasis. Acta Derm Venereol (Stockh) [Suppl 85] 59:39–45
6. Cox DW, Johnson AM, Fagerhol MK (1980) Report of nomenclature meeting for α_1-antitrypsin. Hum Genet 53:429–433
7. Damiano VV, Tsang A, Kucich U, Abrams WR, Rosenbloom J, Kimbel P, Fallahnejad M, Weinbaum G (1986) Immunolocalization of elastase in human emphysematous lungs. J Clin Invest 78:482–493
8. Dubertret L, Bertaux B, Fosse M, Touraine R (1982) Localization of proteolytic activity in psoriatic skin. Br J Dermatol 107:499–504
9. Fräki JE, Hopsu-Havu VK (1976) Human skin proteases. Fractionation of psoriasis scale proteases and separation of a plasminogen activator and a histone hydrolysing protease. Arch Dermatol Res 256:113–126
10. Förster FJ, Neufahrt A, Stockum G, Bauer K, Frenkel S, Fertig U, Leonhardi G (1975) Subcellular distribution of phosphatases, proteinases and ribonucleases in normal human stratum corneum and psoriatic scales. Arch Dermatol Res 254:23–28
11. Gliński W, Zarębska Z, Jabłońska S, Imiela J, Nosarzewski J (1980) The activity of polymorphonuclear leukocyte neutral proteinases and their inhibitors in patients with psoriasis treated with a continuous peritoneal dialysis. J Invest Dermatol 75:481–487
12. Gliński W, Barszcz D, Janczura E, Zarębska Z, Jabłońska S (1984) Neutral proteinases and other neutrophil enzymes in psoriasis, and their relation to disease activity. Br J Dermatol 111:147–154
13. Gliński W, Barszcz D, Jabłońska S, Zarębska Z, Tigałonowa M, Janczura E (1985) Leukopheresis for treatment of psoriasis: Is therapeutical benefit related to reduced activities of neutral proteinases of polymorphonuclear leukocytes? Arch Dermatol Res 278:6–12
14. Hammarström S, Hamberg M, Samuelsson B, Duell EA, Stawiski M, Voorhees JJ (1975) Increased concentration of nonesterified arachidonic acid, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid, prostaglandin E_2, and prostaglandin $F_{2\alpha}$ in epidermis of psoriasis. Proc Natl Acad Sci USA 72:5130–5134
15. Havemann K, Janoff A (eds) (1978) Neutral proteases of human polymorphonuclear leukocytes. Biochemistry, physiology and clinical significance. Urban and Schwarzenberg, Baltimore Munich
16. Heng MCY, Moy RL, Lieberman J (1985) α_1-Antitrypsin deficiency in severe psoriasis. Br J Dermatol 112:129–133
17. Heng MCY, Suni G, Kloss BA (1986) Electron microscopic features in psoriatic patients with α_1-antitrypsin deficiency. J Invest Dermatol 87:59–64
18. Heng MCY, Suni G, Kloss BA, Craig S, Kuehn BS, Chase DG (1986) Significance and pathogenesis of basal keratinocyte herniations in psoriasis. J Invest Dermatol 87:362–366
19. Henseler T, Christophers E (1985) Psoriasis of early and late onset: characterization of two types of psoriasis vulgaris. J Am Acad Dermatol 13:450–456
20. Jeppsson J-O, Laurell C-B, Fagerhol M (1978) Properties of isolated human α_1-antitrypsins of Pi types M, S and Z. Eur J Biochem 83:143–153
21. Lazarus GS, Yost FJ, Thomas CA (1977) Polymorphonuclear leukocytes: possible mechanism of accumulation in psoriasis. Science 198:1162–1163
22. Levin N, Hatcher VB, Lazarus GS (1976) Proteinases of human epidermis; a possible mechanism for polymorphonuclear leukocyte chemotaxis. Biochim Biophys Acta 452:458–467
23. Lipkin G, Gladston M, Kueppers F (1984) Alpha$_1$-antitrypsin deficiency genes: Contributory defect in a subset of psoriatics? J Am Acad Dermatol 11:615–619
24. Mainardi CL, Dixit SN, Kang AH (1980) Degradation of type IV (basement membrane) collagen by a proteinase isolated from human polymorphonuclear leukocyte granules. J Biol Chem 255:5435–5441
25. Miszczuk-Jamska B, Pajdak W (1979) Comparison of the inhibitory activity and concentration of alfa$_1$-antiprotease in normal human sera. Experientia 35:116–117
26. Morse JO (1978) Alpha$_1$-antitrypsin deficiency. N Engl J Med 299:1045–1048
27. Orlov VN, Solod NN, Dulkin IS, Yunusov MA, Radzevich AE (1985) The level of antiprotease activity of the blood

serum and alpha$_1$-antitrypsin in patients with psoriasis. Vestn Dermatol Venerol 2:9–10
28. Perlmutter DH, Cole FS, Kilbridge P, Rossing TH, Colten HR (1985) Expression of the α_1-proteinase inhibitor gene in human monocytes and macrophages. Proc Natl Acad Sci USA 82:795–799
29. Pinkus H, Mehregan AH (1966) The primary histologic lesion of seborrheic dermatitis and psoriasis. J Invest Dermatol 46:109–116
30. Rifkin DB, Crowe RM (1977) Isolation of a protease inhibitor from tissues resistant to tumor invasion. Hoppe-Seyler's Z Physiol Chemie 358:1525–1531
31. Sedgwick JB, Bergstresser PR, Hurd ER (1980) Increased granulocyte adherence in psoriasis and psoriatic arthritis. J Invest Dermatol 74:81–84
32. Sedgwick JB, Bergstresser PR, Hurd ER (1981) Increased superoxide generation by normal granulocytes incubated in sera from patients with psoriasis. J Invest Dermatol 76:158–163
33. Tagami H, Ofuji S (1977) Characterization of a leukotactic factor derived from psoriatic scale. Br J Dermatol 97:509–518
34. Travis J, Johnson D (1981) Human α_1-proteinase inhibitor. In: Colowick SP, Kaplan NO, Lorand L (eds) Methods in enzymology, vol 80, part C. Academic Press, New York London Paris San Diego, pp 754–765
35. Travis J, Salvesen GS (1983) Human plasma proteinase inhibitors. Ann Rev Biochem 52:655–709
36. Vaughan L, Lorier MA, Carrel RW (1982) α_1-Antitrypsin microheterogeneity. Isolation and physiological significance of isoforms. Biochim Biophys Acta 701:339–345
37. Wahba A, Cohen HA, Bar-Eli M, Gallily R (1978) Enhanced chemotactic and phagocytic activities of leukocytes in psoriasis vulgaris. J Invest Dermatol 71:186–188
38. Weiss SJ, Lampert MB, Test ST (1983) Long-lived oxidants generated by human neutrophils: characterization and bioactivity. Science 222:625–628
39. Witt I, Lill H (1984) α_1-Proteinase inhibitor (α_1-antitrypsin). In: Bergmeyer HU (ed) Methods of enzymatic analysis, vol 5. Verlag Chemie, Weinheim Deerfield Beach Basel, pp 448–455
40. Worthington Enzyme Manual (1972) Worthington Biochemical Corporation. Freehold, NJ, pp 132–133, and 125–127

Received November 2, 1987

TAB 6

#15143

RC

JOURNAL OF CELLULAR PHYSIOLOGY 186:73–81 (2001)

Alpha-1-Antitrypsin Stimulates Fibroblast Proliferation and Procollagen Production and Activates Classical MAP Kinase Signalling Pathways

KARIM DABBAGH, GEOFFREY J. LAURENT, ANTHONY SHOCK, PATRICIA LEONI, JENNY PAPAKRIVOPOULOU, AND RACHEL C. CHAMBERS*

Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, Royal Free and University College Medical School, UCL, The Rayne Institute, London, United Kingdom

Connective tissue formation at sites of tissue repair is regulated by matrix protein synthesis and degradation, which in turn is controlled by the balance between proteases and antiproteases. Recent evidence has suggested that antiproteases may also exert direct effects on cell function, including influencing cell migration and proliferation. The antiprotease, α_1-antitrypsin, is the major circulating serine protease inhibitor which protects tissues from neutrophil elastase attack. Its deficiency is associated with the destruction of connective tissue in the lung and the development of emphysema, whereas accumulation of mutant α_1-antitrypsin within hepatocytes often leads to liver fibrosis. In this study, we report that α_1-antitrypsin, at physiologically relevant concentrations, promotes fibroblast proliferation, with maximal stimulatory effects of $118 \pm 2\%$ ($n = 6$, $P < 0.02$) above media controls for cells exposed to 60 μM. We further show that α_1-antitrypsin also stimulates fibroblast procollagen production, independently of its effects on cell proliferation, with values maximally increased by $34 \pm 3\%$ ($n = 6$, $P < 0.01$) above media controls at 30 μM. Finally, mechanistic studies to examine the mechanism by which α_1-antitrypsin acts, showed that α_1-antitrypsin induced the rapid activation of $p42^{MAPK}$ and $p44^{MAPK}$ (also known as ERK1/2) and that the specific MEK1 inhibitor PD98059 totally blocked α_1-antitrypsin's mitogenic effects. These results support the hypothesis that α_1-antitrypsin may play a role in influencing tissue repair in vivo by directly stimulating fibroblast proliferation and extracellular matrix production via classical mitogen-activated signalling pathways. J. Cell. Physiol. 186:73–81, 2001. © 2001 Wiley-Liss, Inc.

α_1-Antitrypsin (α_1-AT) is the major serine protease inhibitor (serpin) in human circulation (Travis and Salvesen, 1983; Perlmutter and Pierce, 1989). It is predominantly produced by hepatocytes, but also by intestinal epithelial cells in vivo (Perlmutter et al., 1989), as well as blood monocytes, pulmonary alveolar cells, macrophages (Perlmutter et al., 1985) and breast cancer cells (Yavelow et al., 1997) in vitro. Studies examining the localised expression of the human α_1-AT gene in transgenic mice also reported the presence of the gene product in the gut, kidney, and lung (Koopman et al., 1989). α_1-AT is an acute-phase reactant and its levels rise sharply by 3 to 4-fold following tissue injury and inflammation (Dickson and Alper, 1974). Its synthesis is regulated by pro-inflammatory cytokines, such as tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) (Takemura et al., 1986; Kalsheker and Swanson, 1990). In addition, endotoxin and neutrophil elastase can stimulate the synthesis of α_1-AT by lung macro-

Abbreviations: α_1-AT, alpha-1-antitrypsin; Hyp, hydroxyproline; IL-6, interleukin-6; NCS, newborn calf serum; MAPkinase, mitogen-activated protein kinase; TGFα, transforming growth factor alpha; TGFβ, transforming growth factor beta; TNFα, tumour necrosis factor-α.

Contract grant sponsor: National Research Council; The Children's Liver Disease Foundation; Contract grant sponsor: National Asthma Campaign; Contract grant sponsor: University College London Medical School; Contract grant sponsor: Wellcome Trust; Contract grant number: 051154.

*Correspondence to: Rachel C. Chambers, Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, Royal Free and University College Medical School, UCL, The Rayne Institute, 5 University Street, London WC1E 6JJ, UK.
E-mail: r.chambers@ucl.ac.uk

Received 22 February 2000; Accepted 3 August 2000

Published online in Wiley InterScience, 30 November 2000.

phages alone or in combination (Perlmutter and Punsal, 1988; Barbey-Morel et al., 1987; Perlmutter et al., 1988).

α_1-AT inhibits a number of serine proteases, but has a much greater affinity for neutrophil elastase. Its main physiological function was ascribed when its deficiency in individuals with α_1-AT gene defects was linked to the premature onset of pulmonary emphysema (Laurell and Erikson, 1963). Normal circulating levels of α_1-AT range between 20 and 53 μM. α_1-AT diffuses through endothelial and epithelial cell walls and is present in the epithelial lining fluid at 10–15% of serum concentrations (Crystal, 1990). Although other serpins are present in the lung, including α_2-macroglobulin and secretory leucoprotease inhibitor (SLPI), α_1-AT contributes to over 90% of the elastase inhibitory activity in the alveolus. If serum α_1-AT levels fall to below 11 μM, the lower respiratory tract is no longer protected and individuals are predisposed to the progressive destruction of alveolar walls and the development of emphysema (Wewers et al., 1987).

In addition to its protease inhibitory functions, α_1-AT has also been reported to influence cell behaviour via both direct and indirect mechanisms. For example, α_1-AT has been shown to both stimulate and inhibit cell proliferation, depending on the cell type and the concentrations examined (Perraud et al., 1988; Scott and Tse, 1988; Hamilton et al., 1990; Graziadei et al., 1994; Yavelow et al., 1997). In addition, α_1-AT-neutrophil elastase complexes and proteolytically modified α_1-AT can act as potent chemoattractants for neutrophils and may therefore play a role in sustaining inflammatory responses at sites of tissue injury (Banda et al., 1988a, b).

The mechanisms by which α_1-AT exerts these cellular effects are poorly understood, although some of its growth-inhibitory effects are thought to be mediated via its ability to inhibit transferrin-receptor binding and cellular iron uptake (Graziadei et al., 1994). In breast cancer cells, α_1-AT is a growth inhibitor and these effects were recently shown to be due to its ability to block the pericellular release of transforming growth factor-alpha (TGFα) by these cells (Yavelow et al., 1997). A cellular receptor for α_1-AT has not been identified to date, although a cell surface receptor termed serpin–enzyme complex receptor (SEC receptor) which binds the α_1-AT-elastase and other serpin–enzyme complexes, and is involved in the internalisation and degradation of these complexes, has been described (Perlmutter et al., 1990a, b; Joslin et al., 1993). In addition, the SEC receptor is thought to mediate the chemotactic effects of α_1-AT–elastase complexes towards neutrophils (Joslin et al., 1992), as well as the induction of α_1-AT synthesis by macrophages (Joslin et al., 1991).

Since α_1-AT deficiency can result in connective tissue destruction, and α_1-AT can exert a number of cellular effects, we hypothesised that α_1-AT may play a role in tissue repair processes by directly activating mesenchymal cells. In order to address this hypothesis, we examined the effect of α_1-AT on fibroblast proliferation and procollagen production in vitro and show that, at physiologically relevant concentrations, α_1-AT is a potent promoter of fibroblast proliferation. α_1-AT was also capable of stimulating fibroblast procollagen production but these effects were not as dramatic as its mitogenic effects. In order to begin to examine the mechanism by which α_1-AT might be acting, we further show that α_1-AT exposure results in the rapid activation of $p42^{MAPK}$ and $p44^{MAPK}$ and that the proliferative effects of α_1-AT can be completely blocked with the specific MEK1 inhibitor PD98059. These data support the hypothesis that α_1-AT may play a role in tissue repair processes by directly stimulating fibroblast proliferation and procollagen production via activation of signalling pathways employed by classical mitogens.

MATERIALS AND METHODS

Materials

The cell lines used in these experiments were human foetal lung fibroblasts (HFL1 and IMR90, American Type Culture Collection, Rockville, MD) and primary human adult dermal fibroblasts (kindly provided by Miss Maddy Parsons, UCL, London, UK). DMEM culture media was from Gibco-BRL (Paisley, UK). Newborn calf serum (NCS) was from Imperial Laboratories, Andover, UK). For most experiments, α_1-AT isolated from human plasma (cat. no. A9024, Sigma, Poole, UK) was used. Salient findings were confirmed with highly purified α_1-AT (cat. no. 178251 Calbiochem-Novabiochem). Both preparations were characterised by SDS-PAGE. The preparation supplied by Sigma was found to contain two major α_1-AT variants in the monomeric form, as well as trace amounts (less than 0.01%) of unidentified high and low molecular weight bands, whereas the preparation supplied by Calbiochem-Novabiochem contained a single α_1-AT variant in the monomeric form and no other detectable bands. In order to remove high concentrations of NaCl (1 M) after reconstitution of the lyophilized powder, this preparation of α_1-AT was extensively dialysed against phosphate-buffered saline (PBS) and DMEM, followed by sterilisation by 0.22 μm filtration, prior to addition to cell cultures.

Determination of cell proliferation

Cell proliferation was assessed using a spectrophotometric assay based on the uptake and subsequent elution of the dye methylene blue according to a previously published method from our laboratory (Oliver et al., 1989). All proliferation experiments were performed on cells in sub-confluent culture in the absence of serum, unless otherwise stated. Briefly, cells were seeded into 96-well plates at a concentration of 4×10^3 cells/well in 100 μl DMEM supplemented with 2% NCS. After 16 h, the medium was removed and replaced with 100 μl fresh serum-free DMEM to quiesce the cells. After this 24-hour pre-incubation, the pre-incubation media was replaced with fresh DMEM or DMEM supplemented with α_1-AT at concentrations between 1 and 60 μM. In some experiments, albumin (Sigma; 1–60 μM) was added to control for non-specific protein effects. Cells were incubated at 37°C for various times up to 72 h ($n = 6$ for each dose and each time point). In order to correct for the absorbance associated with cells present at the onset of each experiment (t_0 absorbance); a plate containing cells plated as described above and left to incubate for the initial 24-hour pre-incubation only was used.

In experiments with pharmacological inhibitors, exposure times and concentrations were optimised so as to avoid inhibiting basal cell proliferation. For tyrosine kinase and MEK1 inhibition experiments, lavendustin A (10 μM), genistein (10 μM) and PD 98059 (50 μM) (all from Calbiochem-Novabiochem, Nottingham, UK) were added at the end of the 24-hour pre-incubation period in serum-free conditions. After 30 min, α_1-AT was added to a final concentration of 30 μM, and the cells were left to incubate for 48 h.

Determination of procollagen metabolism

Cells were grown to confluence in 2.4 cm diameter wells in DMEM supplemented with 5% NCS and pre-incubated in serum-free DMEM supplemented with 50 μg/ml ascorbic acid (BDH, Poole, UK) and 0.2 mM proline (Sigma) for 24 h and exposed to α_1-AT (10 μM and 30 μM) or 40 pM transforming growth factor-β_1 (TGFβ_1; R&D Systems, Abingdon, Oxon, UK) in similar serum-free medium ($n = 6$ for each dose and each time point). Procollagen synthesis and production were assessed after 24 and 48 h exposure according to methods previously described (Chambers et al., 1994). Briefly, the cell-layer and culture medium were harvested and proteins precipitated in 67% (v/v) ethanol at 4°C for 16 h. Ethanol-insoluble proteins (precipitated proteins) were separated from ethanol-soluble free amino acids and small peptides by filtration through a 0.45 μm pore filter (Millipore, Watford, Herts, UK). Ethanol-soluble fractions were dried and both ethanol-soluble and insoluble fractions were hydrolysed in 6 M hydrochloric acid at 110°C for 16 h. Hydroxyproline (Hyp) in hydrolysates was isolated and quantified by reverse-phase-HPLC (Beckman, System Gold, High Wycombe, Bucks, UK). All values obtained were corrected for the amount of Hyp measured in the cell layer and culture medium at the onset of the experiment. Hyp in the ethanol-insoluble fraction represents procollagen production, and Hyp in the ethanol-soluble fraction represents procollagen synthesised and subsequently degraded. Taken together, these two fractions represent the total amount of procollagen synthesised over the incubation period.

Western analysis of phosphorylated proteins

Cells were grown to about 70% visual confluence in 75 cm^2 tissue culture flasks in DMEM-5% NCS, quiesced in serum-free medium for 24 h and exposed to control medium, 30 μM α_1-AT or DMEM-10% NCS for various incubation times. For tyrosine-specific protein phosphorylation experiments, the medium was removed, the cell layer washed twice with HEPES-buffered saline and cells lysed in 1 ml lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 100 mM NaF, 10 mM Na-pyrophosphate) supplemented with fresh 1 mM Na-orthovanadate, 1 mM phenyl-methyl-sulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml leupeptin. An aliquot was assayed for total protein content in a protein assay according to the manufacturer's instructions (Pierce, Chester, UK). Proteins were separated by standard SDS-PAGE (12.5%) in the presence of β-mercaptoethanol in the loading buffer. Proteins were transferred onto Hybond-ECL membranes (Amersham International, Little Chalfont, UK) for 4 h at 250 mA. Equal loading and even transfer were verified by Ponceau S staining (Sigma). For MAPKinase phosphorylation experiments, cells were treated as described above, lysed on ice with lysis buffer (6.2 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue) and electrophoresed by SDS PAGE (10%), followed by transfer to Hybond-ECL membranes.

Tyrosine-specific protein phosphorylation was examined using an anti-phosphotyrosine antibody (R2D2, Roche Bioscience, Palo Alto, CA). Active $p42^{MAPK}$ and $p44^{MAPK}$ were detected using a specific antibody to the activated (phosphorylated) forms of these proteins (New England Biolabs, Beverly, MA). Non-specific antibody binding was prevented by incubating membranes in 5% non-fat dry milk in Tris-buffered saline containing 0.1% (w/v) Tween-20 (TBS-T), for 2 h at room temperature. Membranes were incubated for 1 h at room temperature with the primary anti-phosphotyrosine antibody or overnight at 4°C with the primary antibody to the phosphorylated forms of $p42^{MAPK}$ and $p44^{MAPK}$, followed by a 1-hour incubation with the relevant horseradish-peroxidase-conjugated secondary antibody (DAKO, Bucks, UK) at room temperature. Between and after incubations, membranes were washed three times with TBS-T for 10 min. Immunoreactive bands were visualised by enhanced chemiluminescence (ECL, Amersham International) followed by exposure to KODAK X-OMAT autoradiography film (Sigma) and densitometric scanning.

Statistical analysis

All proliferation and procollagen synthesis data reported are presented as means ± standard error of the mean (SEM) from six replicate cultures and are representative of three separate experiments performed. Statistical evaluation was performed using an unpaired Student's *t*-test—or analysis of variance (ANOVA) using the Neuman–Keuls procedure for multiple group comparisons.

RESULTS

Effect of α_1-AT on fibroblast proliferation

Figure 1 shows the effect of increasing concentrations of α_1-AT on HFL1 cell number after 24, 48, and 72 h at a range of concentrations, where the cell number is based on the spectrophotometric quantitation of the uptake and subsequent elution of the dye, methylene blue. α_1-AT had no effect at 0.1 μM but stimulated fibroblast proliferation in a concentration-dependent manner from 1 μM onwards with absorbance values at 24 h significantly increased by 25 ± 4% ($P < 0.05$), 78 ± 3%, 101 ± 4% and 114 ± 2% above media controls at 1, 10, 30, and 60 μM, respectively ($P < 0.01$). At later time points, values were significantly increased by 36 ± 2%, 58 ± 1%, 82 ± 3% at 48 h and by 62 ± 4%, 97 ± 4%, 118 ± 2% at 72 h for 10, 30 and 60 μM α_1-AT, respectively (all $P < 0.01$). For comparison, 10% serum (NCS) significantly stimulated fibroblast proliferation by 187 ± 5%, 218 ± 4% and 421 ± 13% relative to media control cells at 24, 48, and 72 h, respectively ($P < 0.01$). The validity of the results obtained with α_1-AT was confirmed using a second method in which the cell number was assessed by direct cell counting. In these experiments, 30 μM α_1-AT

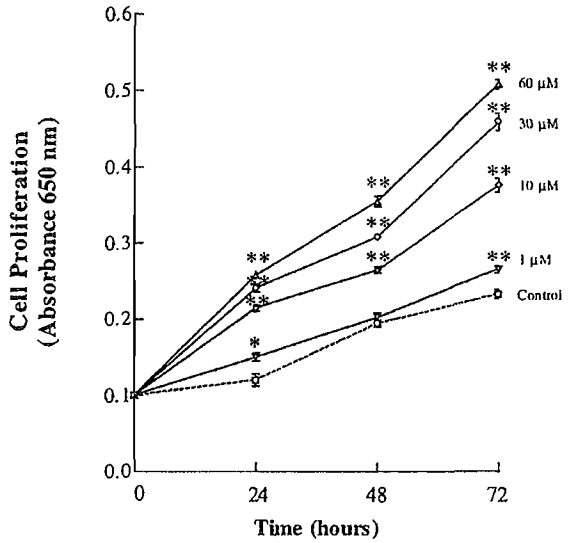

Fig. 1. α_1-Antitrypsin (α_1-AT) stimulates HFL1 proliferation in a concentration- and time-dependent manner. HFL1 cells were incubated in the presence of increasing concentrations of α_1-AT (0.1–60 μM) and cell proliferation was assessed using a spectrophotometric assay after 24, 48, and 72 h. Each value represents the mean ± SEM from six replicate cultures at each time point. Molarities refer to the concentrations of α_1-AT. $^*P < 0.05$, $^{**}P < 0.01$ vs. media control at each time point.

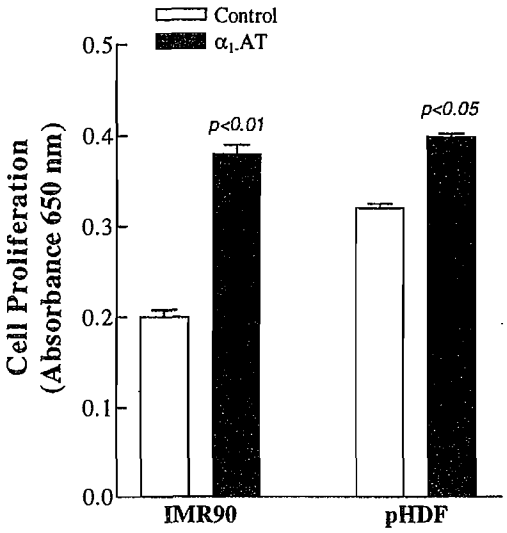

Fig. 2. α_1-Antitrypsin (α_1-AT) stimulates IMR 90 and pHDF proliferation. IMR90 and pHDF cells were incubated in the presence of 30 μM α_1-AT and cell proliferation was assessed using a spectrophotometric assay after 48 h. Each value represents the mean ± SEM from six replicate cultures at each time point. *P* values refer to α_1-AT vs. media control for each cell line.

stimulated cell proliferation by 50 ± 5% at 48 h ($P < 0.01$). To further validate our findings, we also examined the proliferative effects of highly purified α_1-AT obtained from a second commercial source (Calbiochem-Novabiochem). In this experiment, 30 μM α_1-AT obtained from Sigma stimulated HFL-1 fibroblast proliferation, assessed by direct cell counting at 48 h, by 124 ± 13% ($P < 0.01$) above media control values, whereas α_1-AT from Calbiochem-Novabiochem stimulated proliferation by 97 ± 16% ($P < 0.01$). In contrast albumin at all concentrations examined (1–60 μM) had no effect on fibroblast proliferation (data not shown).

In order to ensure that the stimulatory effects of α_1-AT were not restricted to a single cell line, we also assessed the effect of an optimal stimulatory concentration of α_1-AT (30 μM) on the proliferation of a second fibroblast cell line (IMR90), as well as on primary human dermal fibroblasts (pHDF) (Fig. 2) after 48 h exposure. α_1-AT was mitogenic for both cell types examined with values increased by 70 ± 3% ($P < 0.01$) above media controls for IMR90 cells and by 24 ± 2% ($P < 0.01$) for pHDF. For comparison, IMR90 and pHDF were also less responsive to 10% serum than HFL1 cells with proliferation increased by 152 ± 3% ($P < 0.01$) for IMR90 and 101 ± 2% ($P < 0.01$) for pHDF.

Effect of α_1-AT on HFL1 fibroblast procollagen metabolism

The previous results showed that α_1-AT stimulated fibroblast proliferation in a concentration- and time-dependent manner. In order to examine whether α_1-AT affected fibroblast procollagen metabolism, we also assessed the effect of α_1-AT on procollagen production and degradation. Procollagen metabolism experiments were performed on confluent cultures, so that cells were contact-inhibited and not capable of proliferating in response to α_1-AT. However, all procollagen production values have been corrected for cell number at the end of the incubation period. Figure 3 shows the results obtained at two time points and the two doses of α_1-AT examined. Panel A shows the data obtained for procollagen production (Hyp in ethanol-insoluble fraction), whereas panel B shows procollagen synthesis (Hyp in both ethanol-insoluble and ethanol-soluble fractions combined). α_1-AT stimulated fibroblast procollagen production at both concentrations and time points with values increased by 20 ± 3% and 24 ± 2% above media controls (both $P < 0.01$) at 24 h, and by 35 ± 2% and 34 ± 3% (both $P < 0.01$) at 48 h. The trend for procollagen synthesis was similar with values increased by 22 ± 7% and 20 ± 3% (both $P < 0.01$) at 24 h and 30 ± 2% and 26 ± 3% (both $P < 0.01$) at 48 h. At both doses and time points, α_1-AT had no significant effect on procollagen degradation, expressed as an amount of Hyp in ethanol-soluble protein or as a proportion of total procollagen synthesised over the 24- and 48-hour incubation periods. For comparison, an optimal stimulatory dose of TGFβ_1 (40 pM) used as a positive control, stimulated fibroblast procollagen production by 66 ± 7% ($P < 0.01$).

Role of tyrosine kinases in mediating the mitogenic effects of α_1-AT

We next attempted to examine the potential mechanism by which α_1-AT exerts its stimulatory effects on fibroblast function by investigating early signalling

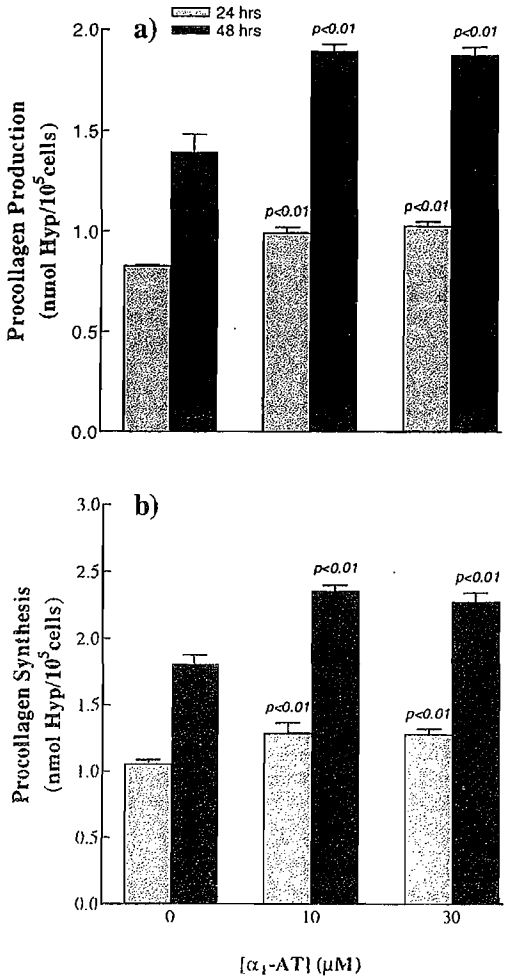

Fig. 3. α_1-Antitrypsin (α_1-AT) stimulates HFL1 procollagen production and synthesis. HFL1 cells were grown to confluence and incubated with 10 and 30 μM α_1-AT for 24 and 48 h. Panel A shows procollagen production and panel B shows procollagen synthesis. Each value represents the mean ± SEM from six replicate cultures at each time point. *P* values refer to α_1-AT vs. media control for each time point.

events induced in response to α_1-AT. We first examined the effects of α_1-AT on tyrosine-specific protein phosphorylation by Western blotting and found that α_1-AT induced the tyrosine-specific phosphorylation of several protein species which included proteins with approximate molecular weights of 40, 45 and 70 kDa (data not shown).

In order to examine the functional role of tyrosine kinases in mediating the stimulatory effects of α_1-AT on fibroblast function, we examined the effect of two tyrosine kinase inhibitors (lavendustin A and genistein) on α_1-AT-induced fibroblast proliferation (Fig. 4). The results obtained showed that at low non-cytotoxic concentrations (10 μM), lavendustin A and genistein

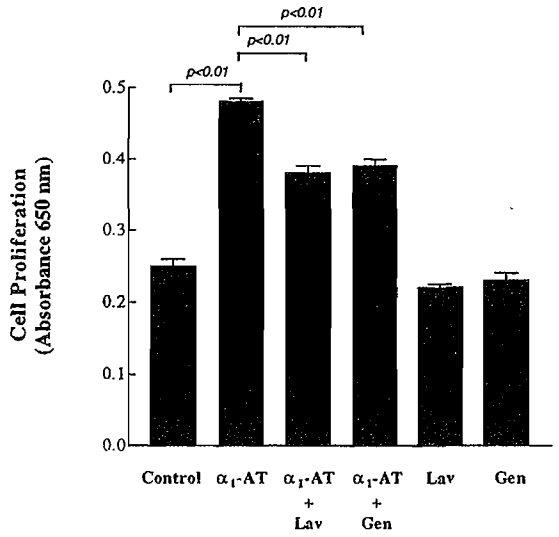

Fig. 4. Tyrosine kinase inhibitors partially block the mitogenic effects of α_1-antitrypsin (α_1-AT). HFL1 cells were pre-treated with 10 μM lavendustin A or 10 μM genistein for 30 min, prior to the addition of 30 μM α_1-AT and cell proliferation was assessed using a spectrophotometric assay after 48 h. Each value represents the mean ± SEM from six replicate cultures at each time point.

abrogated the stimulatory effects of α_1-AT by 44 and 39%, respectively (both $P < 0.01$) with minimal effects on basal cell absorbance values.

Role of MAP kinases in mediating the mitogenic effects of α_1-AT

In light of the finding that some of the proteins on the phosphotyrosine blot had molecular weights which were within the range for the classical MAP kinases, we next went on to examine the role of these kinases in mediating the mitogenic effects of α_1-AT. We first examined $p42^{MAPK}$ and $p44^{MAPK}$ activation by Western blotting using an antibody which specifically recognises the activated forms of these kinases. Figure 5 shows the results obtained for cells treated with 30 μM α_1-AT, 10% NCS or control medium for 5 and 15 min. α_1-AT induced the dramatic phosphorylation of both MAP kinases at both time points examined with comparable effects obtained for cells exposed to 10% serum.

The functional role of these MAP kinases in promoting the mitogenic effects of α_1-AT was again examined by pharmacological inhibition using a selective MEK1

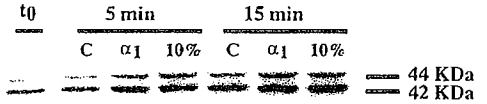

Fig. 5. Effect of α_1-AT on MAP kinase protein phosphorylation. HFL1 cells were incubated with 30 μM α_1-AT, 10% serum or control media for 5 and 15 min. α_1-Antitrypsin (α_1-AT) and 10% serum induced the phosphorylation of both $p42^{MAPK}$ and $p44^{MAPK}$ at both time points examined.

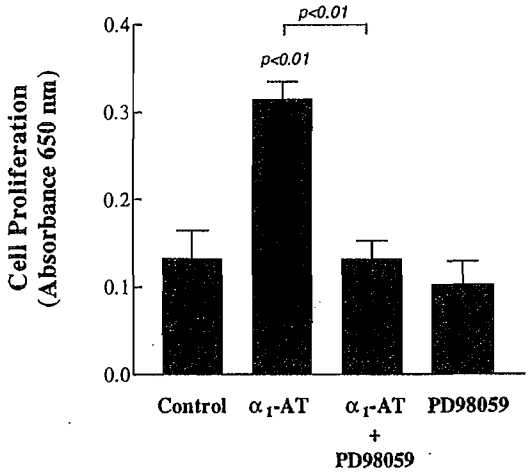

Fig. 6. A MEK1 inhibitor completely blocks the mitogenic effects of α_1-AT. HFL1 cells were pre-treated with 50 μM PD98059 for 30 min, prior to addition of 30 μM α_1-antitrypsin (α_1-AT) and cell proliferation was assessed using a spectrophotometric assay after 48 h. Each value represents the mean ± SEM from six replicate cultures at each time point.

inhibitor (PD 98059) which has been shown to inhibit both $p42^{MAPK}$ and $p44^{MAPK}$ activation. In the representative experiment shown (Fig. 6), 30 μM α_1-AT stimulated fibroblast proliferation by 138 ± 16% ($P < 0.01$) above media control cells after 48 h. The mitogenic effects of α_1-AT were completely blocked for cells exposed to 50 μM PD98059, whereas at this dose PD98059 had no effect on media control cell absorbance values.

DISCUSSION

In this study we examined the hypothesis that α_1-AT may play a role in tissue repair processes by promoting fibroblast proliferation and procollagen production. We show that α_1-AT is a potent promoter of fibroblast proliferation and that these effects are mediated via activation of classical mitogen-activated protein kinases. We further show that α_1-AT also stimulates fibroblast procollagen production but the magnitude of the response is lower than for fibroblast proliferation. This is, to our knowledge, the first report to clearly establish the effect of α_1-AT on fibroblast proliferation and procollagen production, and to begin to investigate the intracellular signalling pathways involved.

Effect of α_1-AT on fibroblast proliferation

The proliferative effects of α_1-AT were confirmed in three different fibroblast lines, including two human foetal lung fibroblast cell lines (HFL1, IMR90) and primary human adult dermal fibroblasts, suggesting that the mitogenic effects of α_1-AT may play a role in a number of tissues. The mitogenic potential of α_1-AT was further confirmed by replicating our original data using highly purified α_1-AT from a second commercial supplier. Analysis of these preparations by SDS-PAGE under reducing and non-reducing conditions showed that for both preparations, α_1-AT was in the monomeric form, so that all of its proliferative effects are likely to be mediated by the monomeric form of the protein rather than other conformational states.

In most of our experiments, fibroblast proliferation was assessed using an extensively employed spectrophotometric assay, based on the uptake and subsequent elution of the dye methylene blue. However, critical results were also confirmed by assessing proliferation by direct cell counting on parallel cell cultures. Although the exact percent stimulation obtained was not identical because of the inherent difference in sample processing between the two methods, a high degree of correlation was always observed. In addition, careful examination of cell cultures by inverted light microscopy at high magnification allowed us to conclude that α_1-AT had no effect of cell morphology.

The mitogenic effects of α_1-AT were both concentration- and time-dependent from 1 μM onwards, with maximal stimulatory effects obtained at concentrations between 30 and 60 μM. These concentrations are within the physiological range for α_1-AT since normal levels of this antiprotease in the circulation range from 20 to 53 μM (Crystal, 1990). Although α_1-AT was mitogenic for all three fibroblast lines examined, α_1-AT exerted its most potent effects on HFL1 cells, for which proliferation was maximally increased by about 138%. This corresponds to approximately a third of the proliferative effect of 10% serum and about half that obtained with an optimal concentration of a classical mitogen, such as PDGF-AB in the assay used. The lowest proliferative response to α_1-AT was obtained with primary adult dermal fibroblasts, with values maximally increased by 25%. However, these cells were only half as responsive to 10% serum as HFL1 fibroblasts, suggesting that they have a lower intrinsic proliferative capacity.

Effect of α_1-AT on fibroblast procollagen metabolism

At concentrations at which α_1-AT stimulated fibroblast proliferation, α_1-AT also promoted HFL1 procollagen production with values increased by about 20% ($P < 0.01$) at the earliest time point examined (24 h). The stimulatory effect obtained was greater at 48 h (35%) but we did not examine further time points because the kinetics of procollagen production for this cell line cease to be linear in serum-free conditions for media control cells (Chambers et al., 1998). The effects of α_1-AT on procollagen production occurred independently of changes in cell number since procollagen metabolism experiments were performed at confluence, and direct cell counts at the end of the 48-hour incubation period showed that α_1-AT did not stimulate HFL1 proliferation at confluence. There was no significant difference in the stimulatory effects obtained with 10 and 30 μM α_1-AT, suggesting that the response in procollagen production was already maximal at 10 μM. Finally, the magnitude of the response obtained with α_1-AT on procollagen production corresponds to about a third of that obtained with an optimal concentration of $TGF\beta_1$, the most potent mediator of procollagen production characterised to date (McAnulty et al., 1991).

We also examined the effect of α_1-AT on intracellular procollagen degradation since this is thought to be another important regulatory step by which some pro-

fibrotic mediators, including TGFβ_1, can increase the amount of procollagen secreted and deposited (McAnulty et al., 1991). In the experiments performed, procollagen production represents the amount of procollagen secreted into the culture medium and deposited in the cell layer during the incubation period. Approximately 20% of all procollagen synthesised basely by HFL1 fibroblasts in serum-free conditions, is degraded intracellularly within minutes of its synthesis (Chambers et al., 1994). Since intracellular procollagen degradation is almost complete and Hyp is derived from the post-translational hydroxylation of proline, Hyp in the ethanol-soluble pool can be taken to represent procollagen synthesised and subsequently degraded during the incubation period. Procollagen synthesis is based on Hyp in the combined ethanol-insoluble and ethanol-soluble protein pools. However, in contrast to TGFβ_1, α_1-AT had no effect on the proportion of newly synthesised procollagen degraded, indicating that all the effects on procollagen production occurred via effects on procollagen synthesis alone.

Role of tyrosine kinases and MAP kinases in mediating the mitogenic effects of α_1-AT

To begin to examine the potential mechanism by which α_1-AT might influence fibroblast function, we assessed the effects of α_1-AT on early cellular signalling events. α_1-AT stimulated the tyrosine-specific phosphorylation of a number of proteins, raising the possibility that α_1-AT may mediate its stimulatory effects on fibroblast function via rapid protein kinase phosphorylation cascades. The functional role of tyrosine phosphorylation was assessed using two general tyrosine kinase inhibitors, lavendustin A and genistein. Lavendustin A is a potent and selective inhibitor of epidermal growth factor receptor kinase (EGFRK) and non-receptor tyrosine kinases such as pp60$^{c\text{-}src}$. Genistein is a widely used general tyrosine kinase inhibitor which also inhibits autophosphorylation of EGFRK but with much lower potency than lavendustin A. Both inhibitors, used at selective concentrations, were effective in blocking the mitogenic effects of α_1-AT by around 40%, suggesting that α_1-AT mediates its effects on fibroblast proliferation via tyrosine kinase-linked signalling pathways.

α_1-AT also induced the rapid phosphorylation and activation of p42^{MAPK} and p44^{MAPK} with comparable effects to those obtained with 10% serum. The functional significance of p42^{MAPK} and p44^{MAPK} activation was investigated by examining the effects of the selective and cell-permeable MEK1 inhibitor PD98059 on HFL1 proliferation in response to α_1-AT. In these experiments, the inhibitor was used at a concentration of 50 μM, since at this dose PD98059 has been shown to be highly selective for MEK1 with no discernible effect on the activation of SAPK1/JNK, p38α, p38γ, p38, PI 3-kinase, PKB and p70 S6 kinase signalling pathways (Alessi et al., 1995). In our experiments PD98059 used at the recommended concentration, completely abrogated the proliferative effects of α_1-AT but had no effect on media control cell proliferation, indicating the critical involvement of the p42^{MAPK}/p44^{MAPK} signalling pathway in mediating the proliferative effects of α_1-AT. We did not attempt to perform similar experiments to assess the role of the p42^{MAPK}/p44^{MAPK} signalling pathway in mediating the stimulatory effects of α_1-AT on fibroblast procollagen production, because we do not believe that the modest stimulation obtained provides a good enough window to block the effect with pharmacological inhibitors. However, the data obtained in proliferation studies are consistent with the involvement of receptor-mediated and classical signalling cascades in transducing the stimulatory effects of α_1-AT on fibroblast function. However, a cellular receptor for α_1-AT has not been identified to date. Studies to identify such a receptor, as well as to establish the potential involvement of the SEC receptor in mediating these effects will form the focus of future studies and should be very informative.

Putative role of α_1-AT in tissue repair processes

Connective tissue formation at sites of tissue repair is regulated by a balance between connective tissue synthesis and degradation. Intense research has lead to the identification of the key regulatory steps in connective tissue synthesis and has highlighted the importance of pro-fibrotic cytokines and growth factors in driving both fibroblast proliferation and matrix protein synthesis (for a review see, Chambers and Laurent, 1997). Connective tissue degradation is thought to be primarily dependent on the coordinate action of proteases, in particular the matrix metalloproteinases (MMPs) and antiproteases (including the tissue inhibitors of metalloproteinases (TIMPs)). Recent evidence has suggested that proteases and antiproteases may also exert direct effects on cell function, including influencing cell migration and proliferation (Chambers et al., 1998 and references therein; Hayakawa et al., 1994). The in vitro data presented in this study demonstrate a novel role for the major circulating serpin in modulating fibroblast proliferation and procollagen production in vitro. These findings represent a significant contribution to the information currently available on the cellular effects elicited by α_1-AT. α_1-AT is present in the circulation at concentrations between 20 and 50 μM and in the pulmonary interstitium at 10–15% of these levels. The circulating levels rise four-fold following tissue injury and inflammation and remain high for up to 6 days which correspond to the circulatory life of the protein (Dickson and Alper, 1974; Crystal, 1989). α_1-AT inhibits the biological activities of neutrophil elastase, which is secreted by activated neutrophils in injured and inflamed areas. Furthermore, α_1-AT can be synthesised by macrophages and pulmonary alveolar cells in response to elastase, α_1-AT-elastase complexes and pro-inflammatory cytokines, such as IL-6, TNFα and TGFβ_1, so that α_1-AT levels can remain elevated in injured tissues.

The results presented in this paper propose a role for α_1-AT in promoting tissue repair processes by directly stimulating fibroblast proliferation and extracellular matrix production at sites of tissue injury. The in vivo relevance of these findings remains to be established, although our observation may be relevant to the findings that topical administration of α_1-AT onto dermal wounds of patients with atopic dermatitis promotes tissue healing in patients who were unresponsive to

steroid treatment (Wachter and Lezdey, 1992). Our results may also be relevant to liver fibrosis in patients with α_1-AT gene defects, in particular individuals who are homozygous for the Z variant. This mutation results in the formation of large hepatic inclusions consisting of polymerised mutant α_1-AT (Lomas et al., 1992). The pathogenesis of liver disease in these patients remains poorly understood, although liver injury and inflammation caused by hepatocyte necrosis is commonly observed. However, it is possible that the cellular effects of α_1-AT described here may also contribute to collagen deposition in this disorder directly, in particular in light of the finding that α_1-AT administration has been reported to accelerate hepatic fibrosis in chronic liver injury models in experimental rats (Ozeki et al., 1989). Finally, α_1-AT augmentation therapy for patients with α_1-AT deficiency and at risk of developing pulmonary emphysema, has been in clinical use for almost 10 years and initial reports suggest that this therapy does prolong life (Mason and Crystal, 1998). Whether this effect is due to the effects on tissue repair, in addition to blocking neutrophil elastase attack of the interstitium, remains at present uncertain.

In conclusion, we have shown for the first time, that α_1-AT is a potent promoter of fibroblast proliferation and a moderate stimulator of procollagen production. Our data support the hypothesis that antiproteases may play a direct role in promoting tissue repair processes at sites of inflammation. Studies to elucidate the mechanisms by which α_1-AT exerts its cellular effects on fibroblast function demonstrated the involvement and rapid activation of tyrosine kinase and MAP kinase signalling pathways raising the possibility that α_1-AT may be mediating these effects via the activation of cell-surface receptors and signalling pathways employed by classical mitogens.

ACKNOWLEDGMENTS

The authors are grateful for the support received from several grant bodies and also acknowledge the support of Roche Bioscience, USA, and the excellent assistance and technical support of Miss Janet Jackman. The authors are also grateful to Dr. Norma Hernandez-Rodriguez for performing initial experiments.

This research was supported in part by the National Research Council and The Children's Liver Disease Foundation (K.D.); National Asthma Campaign (A.S.); University College London Medical School (J.P.); and Wellcome Trust (P.L. and R.C.C.).

LITERATURE CITED

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489–27494.

Banda MJ, Rice AG, Griffin GL, Senior RM. 1988a. Alpha 1-proteinase inhibitor is a neutrophil chemoattractant after proteolytic inactivation by macrophage elastase. J Biol Chem 263:4481–4484.

Banda MJ, Rice AG, Griffin GL, Senior RM. 1988b. The inhibitory complex of human alpha-1-proteinase inhibitor and human leukocyte elastase is a neutrophil chemoattractant. J Exp Med 167:1608–1615.

Barbey-Morel C, Pierce JA, Campbell EJ, Perlmutter DH. 1987. Lipopolysaccharide modulates the expression of alpha-1-proteinase inhibitor and other serine proteinase inhibitors in human monocytes and macrophages. J Exp Med 166:1041–1054.

Chambers RC, McAnulty RJ, Shock A, Campa JS, Newman Taylor AJ, Laurent GJ. 1994. Cadmium selectively inhibits fibroblast procollagen production and proliferation. Am J Physiol 267:L300–L308.

Chambers RC, Laurent GJ. Collagen. 1997. In: Crystal RG, West JB, Weibel ER, Barnes PJ, editors. The lung: Scientific foundations. 2nd Edition. Philadelphia: Lippincott-Raven. p 709–727.

Chambers RC, Dabbagh K, McAnulty RJ, Gray AJ, Blanc-Brude OP, Laurent GJ. 1998. Thrombin stimulates fibroblast procollagen production via proteolytic activation of protease-activated receptor 1. Biochem J 333:121–127.

Crystal RG. 1989. The alpha-1-antitrypsin gene and its deficiency states. Trends Genet 5:411–417.

Crystal RG. 1990. Alpha-1-antitrypsin deficiency, emphysema, and liver disease. J Clin Invest 85:1343–1352.

Dickson I, Alper CA. 1974. Changes in serum proteinase inhibitor levels following bone surgery. Clin Chim Acta 54:381–385.

Graziadei I, Gaggl S, Kaserbacher R, Braunsteiner H, Vogel W. 1994. The acute-phase protein alpha-1-antitrypsin inhibits growth and proliferation of human early erythroid progenitor cells (burst-forming units-erythroid) and of human erythroleukemic cells (K562) in vitro by interfering with transferrin iron uptake. Blood 83:260–268.

Hamilton I, Reynolds GW, Scott GK, Sharfe N, Tse CA. 1990. Effects of human and ovine pancreatic secretory trypsin inhibitors on the proliferation of normal human fibroblasts. Biol Chem Hoppe Seyler 371:79–83.

Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. 1994. Cell-growth promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). J Cell Sci 107:2373–2379.

Joslin G, Fallon RJ, Bullock J, Adams SP, Perlmutter DH. 1991. The SEC receptor recognizes a pentapeptide neodomain of alpha-1-antitrypsin-protease complexes. J Biol Chem 266:11282–11288.

Joslin G, Griffin GL, August AM, Adams S, Fallon RJ, Senior RM, Perlmutter DH. 1992. The serpin-enzyme complex (SEC) receptor mediates the neutrophil chemotactic effect of alpha-1-antitrypsin-elastase complexes and amyloid-beta peptide. J Clin Invest 90:1150–1154.

Joslin G, Wittwer A, Adams S, Tollefsen DM, August A, Perlmutter DH. 1993. Cross-competition for binding of alpha-1-antitrypsin-elastase complexes to the serpin-enzyme complex receptor by other serpin-enzyme complexes and by proteolytically modified alpha-1-antitrypsin. J Biol Chem 268:1886–1893.

Kalsheker N, Swanson T. 1990. Exclusion of an exon in monocyte alpha-1-antitrypsin mRNA after stimulation of U937 cells by interleukin-6. Biochem Biophys Res Commun 172:1116–1121.

Koopman P, Povey S, Lovell-Badge RH. 1989. Widespread expression of human alpha-1-antitrypsin in transgenic mice revealed by in situ hybridization. Genes Dev 3:16–25.

Laurell CB, Eriksson S. 1963. The electrophoretic alpha-1-globulin pattern of serum in alpha-1-antitrypsin deficiency. Scand J Clin Inv 15:132–140.

Lomas DA, Evans DL, Finch JT, Carrell RW. 1992. The mechanism of Z α_1-antitrypsin accumulation in the liver. Nature 357:605–607.

Mason RJ, Crystal RG. 1998. Pulmonary cell biology. Am J Respir Crit Care Med 157:S72–S81.

McAnulty RJ, Campa JS, Cambrey AD, Laurent GJ. 1991. The effect of transforming growth factor beta on rates of procollagen synthesis and degradation in vitro. Biochem Biophys Acta 1091:231–235.

Oliver MH, Harrison NK, Bishop JE, Cole PJ, Laurent GJ. 1989. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J Cell Sci 92:513–518.

Ozeki T, Imanishi K, Ueda H, Uchiyama T, Funakoshi K, Suzuki I, Ohuchi K, Kan M, Satoh T. 1989. α_1-antitrypsin and hepatic fibrosis. Br J Exp Pathol 70:143–152.

Perlmutter DH, Cole FS, Kilbridge P, Rossing TH, Colten HR. 1985. Expression of the alpha-1-proteinase inhibitor gene in human monocytes and macrophages. Proc Natl Acad Sci USA 82:795–799.

Perlmutter DH, Punsal PI. 1988. Distinct and additive effects of elastase and endotoxin on expression of alpha-1-proteinase inhibitor in mononuclear phagocytes. J Biol Chem 263:16499–16503.

Perlmutter DH, Travis J, Punsal PI. 1988. Elastase regulates the synthesis of its inhibitor, alpha-1-proteinase inhibitor, and exaggerates the defect in homozygous PiZZ alpha-1-proteinase inhibitor deficiency. J Clin Invest 81:1774–1780.

Perlmutter DH, Daniels JD, Auerbach HS, De Schryver-Kecskemeti K, Winter HS, Alpers DH. 1989. The alpha-1-antitrypsin gene is expressed in a human intestinal epithelial cell line. J Biol Chem 264:9485–9490.

Perlmutter DH, Pierce JA. 1989. The alpha-1-antitrypsin gene and emphysema. Am J Physiol 257:L147–162.

Perlmutter DH, Glover GI, Rivetna M, Schasteen CS, Fallon RJ. 1990a. Identification of a serpin-enzyme complex receptor on human hepatoma cells and human monocytes. Proc Natl Acad Sci USA 87:3753–3757.

Perlmutter DH, Joslin G, Nelson P, Schasteen C, Adams SP, Fallon RJ. 1990b. Endocytosis and degradation of alpha-1-antitrypsin-protease complexes is mediated by the serpin-enzyme complex (SEC) receptor. J Biol Chem 265:16713–16716.

Perraud F, Besnard F, Labourdette G, Sensenbrenner M. 1988. Proliferation of rat astrocytes, but not of oligodendrocytes, is stimulated in vitro by protease inhibitors. Int J Dev Neurosci 6:261–266.

Scott GK, Tse CA. 1988. Modulation of cell proliferation by protein proteinase inhibitors: A new analytical approach. Biol Chem Hoppe Seyler 369(Suppl):131–135.

Takemura S, Rossing TH, Perlmutter DH. 1986. A lymphokine regulates expression of alpha-1-proteinase inhibitor in human monocytes and macrophages. J Clin Invest 77:1207–1213.

Travis J, Salvesen GS. 1983. Human plasma proteinase inhibitors. Annu Rev Biochem 52:655–709.

Wachter AM, Lezdey J. 1992. Treatment of atopic dermatitis with alpha-1-proteinase inhibitor. Ann Allergy 69:407–414.

Wewers MD, Casolaro MA, Sellers SE, Swayze SC, McPhaul KM, Wittes JT, Crystal RG. 1987. Replacement therapy for alpha-1-antitrypsin deficiency associated with emphysema. N Engl J Med 316:1055–1062.

Yavelow J, Tuccillo A, Kadner SS, Katz J, Finlay TH. 1997. Alpha 1-antitrypsin blocks the release of transforming growth factor-alpha from MCF-7 human breast cancer cells. J Clin Endocrinol Metab 82:745–752.